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Studies during the ongoing grant period demonstrated that: (1) the CTL epitope E75 is partial agonist for activation of CTL effector function. It frequently activates IFN-γ secretion, but infrequently cytolysis. (2) Enhancement of E75 immunogenicity can be achieved by methylene (CH2)-extension at positions in the peptide determined by computer assisted modeling to point upwards and sideways (Alanine position 7). In addition enhanced survival and maintenance of cytolytic activity by E75 can be achieved by priming with the helper peptide G89 (HER-2:776-789) followed by stimulation with CH2-extended E75 (with 3CH2) on the linear side chain of Ala). In addition G89 together with IL-12 (interleukin-2) had a strong IFN-γ inducing effect in lymph nodes of breast cancer patients free of metastasis. G89 alone and IL-12 alone had a much weaker effect. The G89 Th1 (T helper 1) inducing effect was also detected, in metastatic lymph nodes. Together, priming G89 (as helper/conditioner/Th1 inducer) followed by CH2-appended E75 as CTL inducer may form the basis of a novel vaccine approach to breast cancer.

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Constantine G. Tsoumis 10/15/90

PI - Signature Date

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INTRODUCTION

The overall objective of these studies is to develop novel therapeutic approaches to breast cancer by understanding the requirements for successful induction of anti-tumor responses using newly defined T cell epitopes. The use of specific epitopes can overcome limitations in the use of whole self-protein or biased involvement of Th2 cells. The whole protein is not efficient for activation of immune cells. In all studies stronger immune responses were obtained when epitopes were used as immunogens. Breast tumors release tumor Ag in the environment, while antigen presenting cells (APC) macrophages, MΘ, dendritic cells (DC) uptake and represent tumor proteins. However, the disease progresses indicating that this response is inefficient or suppressed. Our recent studies identified an immunodominant CTL epitope on HER-2. Folate binding protein/folate receptor-α (FBP/FR-α) and the aminoenhancer of split (AES) of the Notch complex. We performed an extensive study on HER-2 immunogenicity in healthy donors. We found that only 2/10 donors responded to E75 presented on dendritic cells by induction of cytolytic activity at priming. Three others responded after restimulation. Overall 5/10 donors resonded to E75. This response rate is similar with the one observed in response to melanoma tumor Ag. These results show that the CTL epitopes alone cannot induce a strong CTL response in all patients. For this reason we are currently: (1) using the Th1 subset of $CD4^+$ cells to induce and amplify a CTL response to tumor. The rationale and the reasons for the use of the helper epitopes have been described in detail in the previous reports and are not repeated here and; (2) developing CH2-appended tumor Ag to increase the immunogenicity of E75 (used as a model).

Studies during the previous year have identified a dominant HER-2 epitope recognized by $CD4^+$ cells designated as G89 = HER-2 (777-789) SPYVSRLL GICL.T. This epitope

induced proliferation and type 1 cytokines from PBMC of breast cancer patients, and healthy donors, suggesting that precursors for HER-2 specific Th1 $CD4^+$ cells exist

A second objective of this study was to characterize the responsiveness and the type of response of cells in the lymph nodes of patients with breast cancer to G89. Breast disease spread through the lymph nodes. Thus, the availability of an antigen which will condition the LN environment towards Th1 should be important for delaying the spread of breast cancer to LN. A third objective was to obtain a preliminary estimate of $G89^+$ cells. We are currently addressing this question. Preliminary results to this question show that the $G89^+$cells are present in small numbers, but they are very high producers of IFN-γ.

A third objective of this study was to determine whether G89 provides help (although not yet clearly defined) for the ultimate goal which is CTL induction. Studies on this direction are ongoing and preliminary results are available. These studies emphasize the ability of priming with G89 (as an approach to APC conditioning) in induction of higher cytolytic activity. Three systems are used for this analysis: (1) healthy donor PBMC; (2) DC1S PBMC and (3) LN cells. We found that priming with G89 increased the number of lytic effectors specific for E75, after restimulation with E75. We also found that priming with G89 increased the number of lytic effectors at restimulation with the CH2-appended CTL epitope E75 (A7.3). The lytic effectors in the second case were of high avidity for E75 25-50 nM (100 fold/2 log). Although this level of sensitivity is still below the optimal desired (0.1 – 1 nM pulsed) this is a significant increase over the previously detected levels.

Studies in CTL induction in LN and the use of G89 to potentiate this response are ongoing. In conclusion, our studies and results during the last year identified two key points: (1)

G89-priming and (2) CH2-appendage, where the immune response to breast cancer Ag is amplified and its affinity for the target increase.

PROGRESS REPORT

MATERIALS AND METHODS

Cells, Antibodies and Cytokines. HLA-$A2^+$ PBMC were obtained from healthy volunteers from the Blood Bank of M.D. Anderson Cancer Center. The HLA phenotypes of the donors used in this study were: Donor 1 (A2, B7, 44), Donor 2 (A2, 33, B40, 44), donor 3 (A2, 33, B41, 81), Donor 4 (A1, 2, B27, 44), Donor 5 (A1, 2 B44, 57, Cw5, 6), Donor 6 (A2, 31, B35, 44, Cw4, w5). For the other four donors only the HLA-A2 expression was determined. T2 cells, ovarian SKOV3, SKOV3.A2 cells, and indicator tumors from ovarian ascites were described). mAb to CD3, CD4, CD8 (Ortho), CD13 and CD14 (Caltag Laboratories, San Francisco, CA), B7.1 and B7.2 (CD80 and CD86, Calbiochem), ICAM-1 (ICAM-1 (CD54, Calbiochem), CD40L (Ancell, Bayport, MN), HLA-A2 (clone BB7.2, ATCC), and MHC-II (L243, DAKO Corp., Carpintera, CA) were used as unconjugated, FITC, or PE conjugated. The following cytokines were used: GM-CSF (Immunex Corp., Washington, DC), specific activity 1.25 x 10^7 CFU/250 mg; TNF-*a* (Cetus Corp., Emeryville, CA), specific activity 2.25 x 10^7 U/mg, IL-4 (Biosource International), specific activity, 2 x 10^6 U/mg; IL-2 (Cetus Corporation) specific activity of 18 x 10^6 IU/mg. IL-12 at 5 x 10^6 U/mg was a kind gift from Dr. Stanley Wolf, Department of Immunology, Genetics Institute, Cambridge, MA.

Synthetic Peptides. The HER-2 peptides used were: E75 (369-377) and the unnatural modified Muc-1 peptides D125: (GVTSAKDTRV) and D132 (SLADPAHGV). The corresponding natural peptides do not bind HLA-A2. Introduction of an HLA-A2 anchor and sequence modification in Muc1 in residues contacting TCR lead to an unnatural sequence (12). All peptides were prepared by the Synthetic Antigen Laboratory of M.D. Anderson Cancer Center and purified by HPLC. Peptides were 95-97% pure by amino acid analysis. Peptides

were dissolved in PBS and stored frozen at –20 °C in aliquots of 2 mg/ml. Polyglycol bead containing E75 were a kind gift of Dr. Kenneth Grabstein (Corixa Corporation). The eight HER-2 peptides constructed by the Synthetic Antigen Laboratory of M. D. Anderson Cancer Center derived from the aminoacid sequence of the human HER-2. These peptides have been previously selected based on the computer program ANT.FIND.M. which was used for the prediction of candidate T cell epitopes on HER-2 protein. The sequences of peptides used in this study are as follows: D122(396-406): QLQVFETLEET, F12 (449-465) GISWLGLRSRELGSGL; G88 (450-463); ISWLGLRSRELGS; F7 (776-789) GSYVSRLLGICL; G89 (777-790); SPYVSRLLGICL; F13 (884-899) VPIKWMALESILRRRF; G90 (886-898); IKWMALESILRRR and F14 (474-487): TVPWDQ LFR NPHQA. The residues that are potential HLA-DR11 anchors are underlined. Because of the ability of peptides to bind on alternative registers, as well as of mutations in the MHC-class II binding pocket, motifs that predict specific binding to MHC-class II are not yet well defined. Most of these HER-2 peptides contain a minimum of two of the three major anchors reported for the major HLA-DR types (i.e. HLA-DR4, -DR1, -DR11). A larger panel of HER-2 peptides was previously tested for the ability to induce proliferation of PBMC from randomly selected healthy donors and ovarian cancer patients. Results of our previous study indicated that F7 and F13 induced proliferative responses of PBMC of healthy donors (57% and 62%, respectively) and cancer patients (24 and 21%, respectively) with higher frequency than the other HER-2 peptides tested. In contrast F12 was less frequently recognized (21% in healthy donors and 4% in cancer patients). F7, and mainly his analog G89 (777-790) induced proliferation of PBMC from a large number of HLA-DR4+ breast cancer patients. The HER-2 intracellular (ICD) and extracellular

domains (ECD) were gifts from Dr. Kenneth Grabstein, Corixa Corporation and were prepared as described.

Generation of PBMC-Derived DC. $CD13^+$ DC were generated from freshly isolated PBMC following the established CD14 methods. Complete RPMI medium (containing 10% FCS) supplemented with 1000 U/ml GM-CSF and 500 U/ml IL-4 was added to each well containing plastic-adherent cells, changed every 48 h and maintained for 7 days. In separate studies, performed in parallel, we attempted to grow DC in media containing either HS, or in AIM-V medium. The growth and expression of surface markers of DC cultured in complete RPMI medium was significantly better than of DC cultured in other conditions thus in this study only DC cultured in complete RPMI medium were used. $CD8^+$ cells were isolated by removing first the $CD4^+$, and then the $CD16^+$ and $CD56^+$ cells from the nonadherent population using Dynabeads (Dynal, Oslo, Norway). After depletion, the resulting cells were 97% $CD8^+$ as determined by flow cytometry.

T Cell Stimulation by Peptide Pulsed DC. DC were washed three times in serum-free medium, plated at 1.2×10^5 cell/well in 24-well culture plates, and pulsed with peptides at 25-50 μg/ml in serum-free medium for 4 h before addition of responders. TNF-α (50 U/ml) was added to DC for the last hour to stimulate Ag uptake and presentation. Autologous PBMC or isolated $CD8^+$ cells in RPMI 1640 containing 10% human serum (HS) were added to DC at 1.5×10^6/ml, followed 60 min later by IL-12 at 3 IU/ml. IL-2 was added 16 h later to each well at 60 IU/ml and every 48 h thereafter. For inhibition studies, mAb specific for B7.1, B7.2, HLA-A2 were added to DC 1 h before responders in amounts reported to be inhibitory by the manufacturers.

CTL and Cytokine Assays. Recognition of peptides used as immunogens by CTL was performed as described. Equal numbers of viable effectors from each well were used in all assays. To minimize cross-reactive recognition of human peptides, all stimulations were preformed in media containing HS while CTL assays were performed using media containing FCS (15). This minimized background cytolysis due to activation of other autoreactive cells to Ag present in HS. Specific lytic units (LU) were determined as described and are expressed as LU $30/10^7$ cells. The ability of PBMC to release cytokines in response to stimulation by peptides activating $CD4^+$ cells was determined by culturing PBMC either as unstimulated (NP) or stimulated with the corresponding peptides. Supernatants were collected after 48 h and stored frozen at $-20^{\circ}C$ until assayed for cytokine levels. IFN-γ, IL-4 and IL-10 were measured by double sandwich ELISA using the corresponding kits provided by Biosource International (Camariyo, CA). The assays were calibrated with human recombinant IFN-γ, IL-4 and IL-10 to detect each cytokine in the range of 10,000 pg/ml.

Determinant Spreading Studies. Peripheral blood was collected from two healthy donors designated as donor 1 (HLA-A2, 23, B7.48, DR7, 11, DQ2.6), donor 2 (HLA-A11, 68, B51, 67, DR13, 14, DQ5, 6) and from an ovarian cancer patient, designated as patient 1 (HLA-A24,, 28, B35, W6, 70, CW3, 4, DR11, 14, DQ5). The HLA-typing for the healthy donors was performed in the Blood Bank of the M. D. Anderson Cancer Center, while the typing of the patient was performed using molecular methods. Peripheral blood mononuclear cells (PBMC) were separated by Ficoll-Hypaque and used for stimulation immediately after separation.

T Cell Proliferation Assays. For characterization of T cell responses to HER-2 peptides, PBMC were plated into 24 well plates at a final concentration of 2 X 10^6/ml in complete RPMI medium supplemented with 5% human AB serum. Each peptide was added at a

final concentration of 20 μg/ml. Five and six days later, equal volumes of cells were plated in tetraplicate in a 96 well plate, incubated with 1 μCi of tritiated thymidine (3H-Tdr) (Amersham) and counted as previously described (12,13). Results are expressed as stimulation index (S.I.) representing the ratio between the mean c.p.m. of the cultures stimulated with peptide, and the mean c.p.m. of the cultures that have not been stimulated with peptide (N.P.). For *in vitro* expansion of T cell cultures, six days after the primary stimulation, IL-2 (Cetus) was added in each culture at a final concentration of 20 Cetus U/ml for four – five additional days. Afterwards, over the next five days, IL-2 was gradually removed from these cultures. For the last 48 h before restimulation, the lymphocytes were rested by being cultured in the absence of exogenous IL-2. Restimulated experiments were performed in same way as primary stimulation with the difference that APC were X-Ray irradiated (10,000 Rads) autologous PBMC.

Induction of Cytolytic Activity in the Peripheral Blood of Healthy Donors by the HER Peptide E75

The presence of tumor reactive CTL in tumor infiltrates and in the peripheral blood of cancer patients demonstrates an immune response against tumors which apparently cannot control disease spread. This raises concerns as to whether amplification of this response may be useful during disease progression. Induction of tumor-reactive CTL in healthy donors at risk, as well as in patients free of disease may, be therapeutically important, based on the hypothesis that CTL which recognize tumors early may be more effective in containing their progression than CTL that expand only when the disease progresses. To address the feasibility of priming cytolytic activity in healthy donors, we used the HER-2 peptide E75 (369-377) as immunogen and autologous peripheral blood mononuclear cells (PBMC) derived dendritic cells (DC) as antigen presenting cells (APC). We found that out of ten health donors tested, two responded at priming with E75 presented on autologous DC by induction of E75-specific CTL activity. Three other responders were identified after two additional restimulations. Of these 5 responders, three recognized E75 presented on the ovarian tumor line SKOV3.A2 as demonstrated by cold-target inhibition experiments. Induction of cytolytic activity at priming was enhanced in responders by TNF-α and IL-12 but not in the non-responders. αB7.1 mAb added at priming enhanced induction of lytic activity in only one of the four non-responding donors tested, suggesting that in the majority of donors, E75-precursor CTL were not tolerized. Because of the possibility that disease may develop in non-responders, strategies to improve the immunogenicity of tumor Ag for healthy donors may be required for development of cancer vaccines. This point started to be addressed in these studies (please see "Section 6B, p.15.

2. In addition to studies on cytolysis (completed and published between these two reports) annual additional studies indicate that E75, as well as all other tumor epitope CTL epitopes are weak and partial agonists for $CD8^+$ T-cell activation. The partial agonism is characterized by the ability of tumor Ag to induce IFN-γ, IP-10, and small levels of IL-12 at stimulation with the peptide presented on dendritic cells (DC). These effects are rapid and evident at priming within less than 24 h. However, the same peptide is incapable of activating the lytic activity at priming in the majority of the same donors. This lead to studies to enhance the agonistic activity of E75 by methylene extension.

Induction of Determinants Spreading by HER-2 Epitopes Recognized by $CD4^+$ Cells. Immunization with tumor antigens induce cellular and humoral immune responses. These responses by T cells are specific for defined eiptopes (determinants) in the molecule of the immunizing tumor Ag. Expension of such responses to self-Ag requires induction of autoimmunity to tumor. According with systems of autoimmune disease, expression of T cell autoimmunity is characterized by diversification of responses from the inducer determinant to other responder (cryptic) determinants. Since similar strategies may be useful for therapy of human cancers, we investigated whether induction of response to a HER-2 peptide F7 (776-789) induces enhanced reactivity of other HER-2 peptides. We found that stimulation with F7 can expand a response to another epitope F13 (884-899) in both on ovarian cancer patient with progressive disease and a healthy donor which shared HLA-DR11. This response was characterized mainly by increased IFN-γ secretion, and proliferation, but was not observed with another donor which shared HLA-DR14, and HLA-DQ5 with the patient. Since repeated vaccination with the same epitope may lead to decline of primary cell reactivity due to apoptosis spreading of the response to other epitopes in the tumor Ag may provide an approach for

maintaining an inflammatory Th1 response during cancer vaccination. These studies are now completed and published *(Cancer Immunology Immunotherapy, 49:00-00, 2000, in press).*

4. Axillary lymph nodes (LN) cellular immune responses to HER-2/neu peptides in patients with carcinoma of the breast. The studies performed this year completed the studies initiated during previous years. We demonstrated that HER-2 peptide G89 induced Th1 responses not only in the Metastasis (-) LN but also in the Metastasis (+) LN. The terms Tum^+ and Tum^- Ln used in previous reports have been replaced with metastasis (+/-) = Mets (+/-) at the recommendation of Dr. Henry Kuerer. Indeed, the presence of breast tumor in LN indicate metastasis. Although this part of the project was delayed because of personnel changes, this part of the study is now being completed. *An abstract presenting our results has been submitted to the Society of Surgical Oncologists on 8/30/00 (Kuerer et al., attached).* Some further analyses are being performed to complete the manuscript for publication.

5. Characterization of the Mechanism of Help by G89. Studies during previous years demonstrated that T cell help is required for induction of tumor-specific CTL. To characterize the mechanisms of T cell help we initiated studies to address the question whether G89 alone on together with E75 enhance IL-12 production. The results in **Figure 1** confirm the results of the previous study that G89 and the CH2-extended E75 have an additive effect on IL-12 secretion. Thus G89 by itself does not have a significant IL-12 inducer effect.

To address the question as to whether G89 conditioned DC for higher IL-12 secretion. DC were pulsed with various peptides followed by addition of isolated PBMC from the same donor. Two days later, the non-adherent cells were removed and DC were pulsed with E75 followed by addition of freshly isolated nonadherent PBMC from the same donor. These PBMC were not previously co-cultured with DC. The results in **Figure 2** show that G89 conditioning of

autologous DC induced high levels of IL-12 from DC at encounter with T cells. These levels were 2-3 fold higher that the ones induced by E75 alone or in response to E75 conditioning followed by E75 stimulation. Thus, it appears that G89-conditioning increased the ability to DC to respond to E75. To address the question whether this impacted on the induction of CTL activity the responders were tested for recognition of E75 on T2 cells. The results in **Figure 2B, C** show that G89 conditioning increased the numbers of E75-effectors by almost 60% (from 196 to 313 LU). This effect was G89-specific, in that the conditioning with G90 increased the number of CTL effectors by only 26% compared with the effectors induced in the presence of E75 conditioning. Thus one of the helper effects of G89 appears to be associated with an increase in the overall number of the effectors at stimulation with E75/A7.3 although this may represent an increased survival of the effectors or an increase in the numbers of effectors. Ongoing studies are addressing this point.

6. Characterization of the Effects of G89 Stimulation on the Induction of Cellular Responses to E75. This work was performed in breast cancer patients with Ductal Cell Carcinoma *in situ* (DCIS). This group of patients allowed to obtain blood in more than one occasion, thus a more complete study could be performed.

(a) The G89 expanding cells by proliferation constitute a small fraction of the PBMC population in DCI3 patients.

To address the question of the presence and ability of G89+ cells to proliferate, PBMC from donor DCIS-1 were labeled with carboxyfluorescein (CFSE) and stimulated with G89, G90, or without peptide as a control. The results in **Table 1** show that the number G89 responding cells which underwent two divisions was only 0.57% higher than the NP-stimulated cells. In the same experiment G90+ cells which underwent two divisions were 2.88% higher

than the number of control cells which were not stimulated with peptide. Therefore, compared with T cells which were not antigen stimulated, the original $G89^+$ cells were 0.143% (0.57:4) while $G90^+$ cells were 0.720% (2.88:4). This represents 143 $G89^+$ cells per 10^6 cells and $720/10^6$ $G90^+$ cells, respectively. Thus G89-responding cells are present in the blood of DCIS patients at lower frequencies than the cells responding to G90. To address the question whether G89 induced a Th1 response in this patient, we determined the levels of IFN-γ and IL-10 induced by G89 at priming. The results show the IFN-γ response to G89 increased in the first 48-72 h, than remained stable while the IL-10 response decreased in the same time. The G90 responding cells showed significantly lower levels of IFN-γ response in the same assay. They also showed borderline levels of IL-10 response. It should be noted that calculating the IFN-γ/IL-10 ratios, this may indicate a higher IFN-γ/IL-10 ratio for G90 than for G89. However, on a per cell basis the IFN-γ/IL-10 levels were similar (G89=13:3.5, vs G90 = 0.77:0.17). The fact that the overall IFN-γ response was substantially higher to G89 than to G90 (approximately 17-fold) suggested that G89 may be the helper Ag of choice to induce Type 1 conditioning in this patient. It should be also mentioned that on a per cell basis the overall IFN-γ response was also higher to G89 than the G90 response with respect IL-10. However, the overall levels of both IFN-γ and IL-10 were very low in response to G90 compared to G89. These results do not exclude G90 as a helper epitope, but indicate that although $G90^+$ cells are present and proliferate their effector cytokine response is low. Since responses to Ag such as G89 and G90 are MHC-restricted it is possible that in other patients G90 will be the Th1 epitope of choice.

(a) Priming with G89 Enhance Induction of High-Affinity CTL for E75 at Stimulation with Methylene Modified E75. The studies described below are ongoing and preliminary results are shown to indicate the progress of this research.

(1) To enhance the T cell activating ability of E75 and of other tumor Ag, we are currently developing a novel class of immunogens. These immunogens are based on the principle that TCR activation depends on (a) the affinity of TCR for the peptide and; (b) the ability of the peptide to modulate the TCR intracellular signaling. The development of this class of tumor Ag is based on the principle that introduction of short (2-4) CH2 (methylene) extensions in the side-chain of aminoacids pointing upwards and sideways (towards the TCR) should increase the affinity of the peptide for TCR, because of the formation of van der Waals bonds. Since van der Waals forces are weak they can be gradually increased or decreased by addition of CH2 groups. This approach should enhance the Ag stimulating ability with regard cytokine production and cytolysis. Based on this principle E75 analogs were developed with CH2 extensions in the Ala 7 of E75 (Ala) points sidewasy. They are as follows: = E75 (A7.0) (wild-type) no extension 1CH2 extention: A7.1 = Ala-7 replaced with aminobutyric acid, 2CH2 extensions A7.2 = Ala7-replaced with norvaline and 3 CH2 extensions: A7.3 = Ala7-replaced with norleucine. All these three aminoacids have a linear CH2 side chain which extends the Ala side chain. They differ in this regard from the natural: saline, leucine, isoleucine which have branched side chain. Preliminary studies indicate that they are capable of inducing significantly higher levels of IFN-γ from T cells than E75 **(Figure 3)**. To address the question whether priming with G89 is essential for high-avidity CTL induction, non-adherent PBMC from this DC15 patient were primed with NP, G89, and G90 followed by restimulation one week later with NP, G89 + E75, G90 + E75. The result in **Figure 4** show that stimulation with G89 and particularly with G90 induced a significant increase in the overall cell numbers compared with NP + IL-2. One week later after restimulation (two weeks after priming) the number of recovered cells start decreasing but the number of live G89 and G90 stimulated cells was still

higher. To address which of the G89 and G90 induce higher helper activity for cytolytic activity, the surviving cells, were tested for recognition of the wild-type Ag (E75) in a CTL assay. We focused only on high avidity CTL: High avidity CTL should recognize the exogenous pulsed peptide at concentrations $\leq 10^{-7}$ M. Low affinity CTL described in the previous studies as well as in this report **(Figure 2)** recognize E75 at exogenous pulsed concentration of 10^{5}M. (10-25 μg/ml). Thus, there is a log increase in sensitivity of these CTL for the wild-type Ag. The results in **Figure 4B** show that CTL derived from cultures primed with G89 and restimulated with A7.3 expressed higher levels of lysis of targets pulsed with 100 nM of E75.

Key Research Accomplishments.

(1) Demonstration of the fact that CTL epitopes act as weak and partial agonists for stimulation of cytolysis, although they are strong inducers of IFN-γ.

(2) Demonstration of the fact that the CD4 recognized epitope acts as a Th1 inducer in the metastatic lymph nodes of patients with breast cancer. This is an important accomplishment, because it provides for the first time an antigen which can be used for immunomodulation even prior the vaccine, to CTL activation to reverse the Th2 environment in metastatic lymph nodes.

(3) Development of CH2-appended tumor Ag, which are stronger stimulators than E75 for IFN-γ and cytolysis.

Reportable Outcomes. We apologize for not providing this section last year. At that time there was a somewhat interim period for completion of experiments and manuscript submission. At this time a part of our results were published or they are in press and the results are listed below:

(1) We demonstrated that E75 from (HER-2) as well as other CTL epitopes from other tumor epitopes act as weak and partial agonists with respect activation of T cells from healthy donors

and breast cancer patients. This partial agonism is characterized by induction of IFN-γ of IL-10 and of low levels of IL-12 and IL-2. There is no induction of IL-4, and significantly weaker induction of IL-10 than IFN-γ.

(2) These CTL epitopes are weak inducers of cytolytic activity. Only 2/10 donors and a similar proportion of patients (the study is under completion) activated lytic function at priming with E75 presented on autologous DC, regardless of the supporting cytokines (IL-12, TNF-α, IL-2 as well as α-IL-10, αIL-4, αTGF-β). The conclusions of these studies have been published *(Oncology Reports, 7:455-466, 2000, J. Interferon and Cytokine Research, 20:391-401, 2000, Clin. Cancer Res., 6:00-00, 2000, in press).*

(3) The result of one of these studies (Clin. Cancer Res., 6:00-00, 2000.) also supports the hypothesis that the stimulation systems may be identifying individuals which will respond to a vaccine with a tumor Ag, considering that the active epitope used is the wild-type epitope. This may have implications for preventive vaccination in high risk individuals: 2/10 responded at priming, 5/10 responded after re-stimulation, but 5/10 did not respond. The implications between this responsiveness, population studies and HER-2 overexpression should be the focus of future studies.

(4) Demonstration of determinant spreading for induction of Th1 responses, using HER-2 peptides recognized by $CD4^+$ *(Cancer Immunology Immunotherapy, in press 2000).*

(5) Demonstration of the Th1 inducing effect of G89 (HER-2, 777-789) in the metastatic axillary lymph nodes of patients with breast cancer. Taking in consideration the helper dependency of DNA vaccines and of vaccines delivered by various vectors (e.g. adenovirus) G89 can be used for priming before the vaccine itself to modify the cellular environment in the lymph node.

Table I. $G89^+$-cells represent a small fraction in the T cells of the DCIS patient.

Cell divisions	% Cells in each $CFSE^+$ peak (stimulating antigen)		
	N/P (none)	G89	G90
0	47.89	50.08	47.71
1	45.25	42.50	42.61
2	6.85	7.42	9.73
Specific increase (%)	0	0.57	2.88

PBMC from the DCIS patient 1 were stimulated with each peptide at 20 μg/ml for 6 days. Cells were labeled with CFSE prior to stimulation. FACS analysis was performed without additional staining and the numbers of cells in each $CFSE^+$ peak were determined. The % positive cells were determined by dividing the number of cells in each peak by the total number of $CFSE^+$ cells counted.

Legends to the Figures.

Figure 1. Induction of IL-12 by CH2-extended E75 (3CH2 groups added at the side chain of Ala7) alone (A) or together with G89.

Figure 2. Pretreatment (conditioning) of dendritic cells at priming with G89 leads to higher levels of IL-12 and higher numbers of E75-specific CTL after restimulation with E75. (A) IL-12 levels; (B) (C) Cytolytic activity.

Figure 3. High levels of induction of IFN-γ by CH2-extended E75 in a donor 3 with low responsiveness to E75 with responsiveness to E75. Responders were plastic non-adherent PBMC. Stimulators were autologous DC cultured in GM-CSF IL-4. Immunogens were: E75: A7.2 (E75+2CH2) at Ala7, A7.3 (E75+3CH2 at Ala7) K66: E75+1CH2 at Ser 5. IFN-γ was determined using an ELISA kit.

Figure 4. Induction of IFN-γ and IL-10 in T cells from DCIS patient at priming with G89 and G90.

(A) IFN-γ induction; (B) IL-10 induction; (C) IFN-γ/IL-10 ratios. Supernatants were collected at the indicated times and the cytokine content was determined by ELISA (as described in the Materials and Methods). IL-12 was not added in this experiment. Note the increase in IFN-γ in the 72-96 h interval with corresponding decrease in IL-10.

Figure 5. Priming with G89 of PBMC from a DCIS patient leads to higher recovery of T cells after restimulation with G89 + A7.3 (A) and higher numbers of E75-specific high avidity CTL (B). % viable cells on day 13 was calculated in relation to % viable cells on day 7.

FIGURE 1

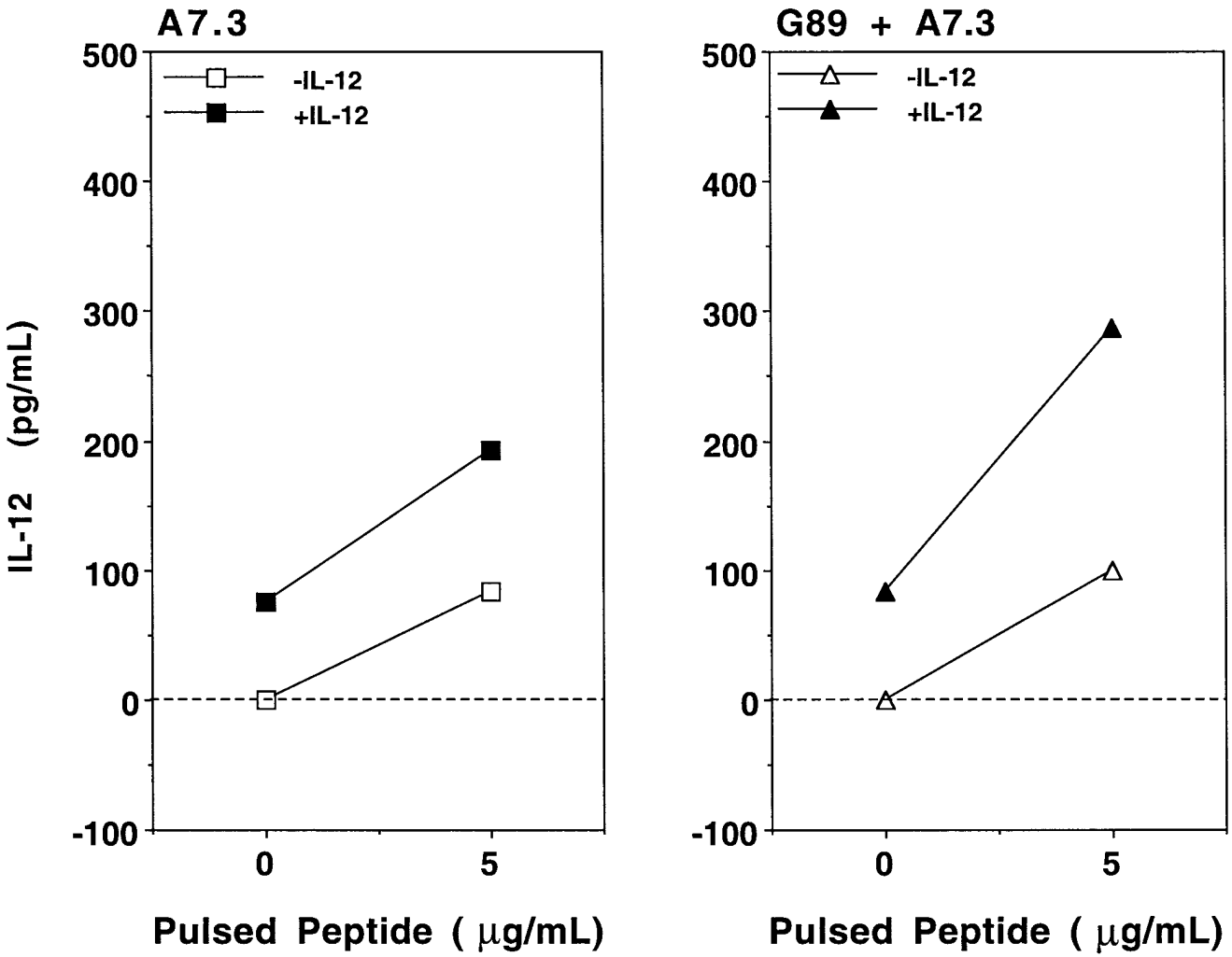

A7.3
G89 + A7.3
-IL-12
+IL-12
500
400
300
200
100
0
-100
IL-12 (pg/mL)
0
5
Pulsed Peptide (μg/mL)

FIGURE 2

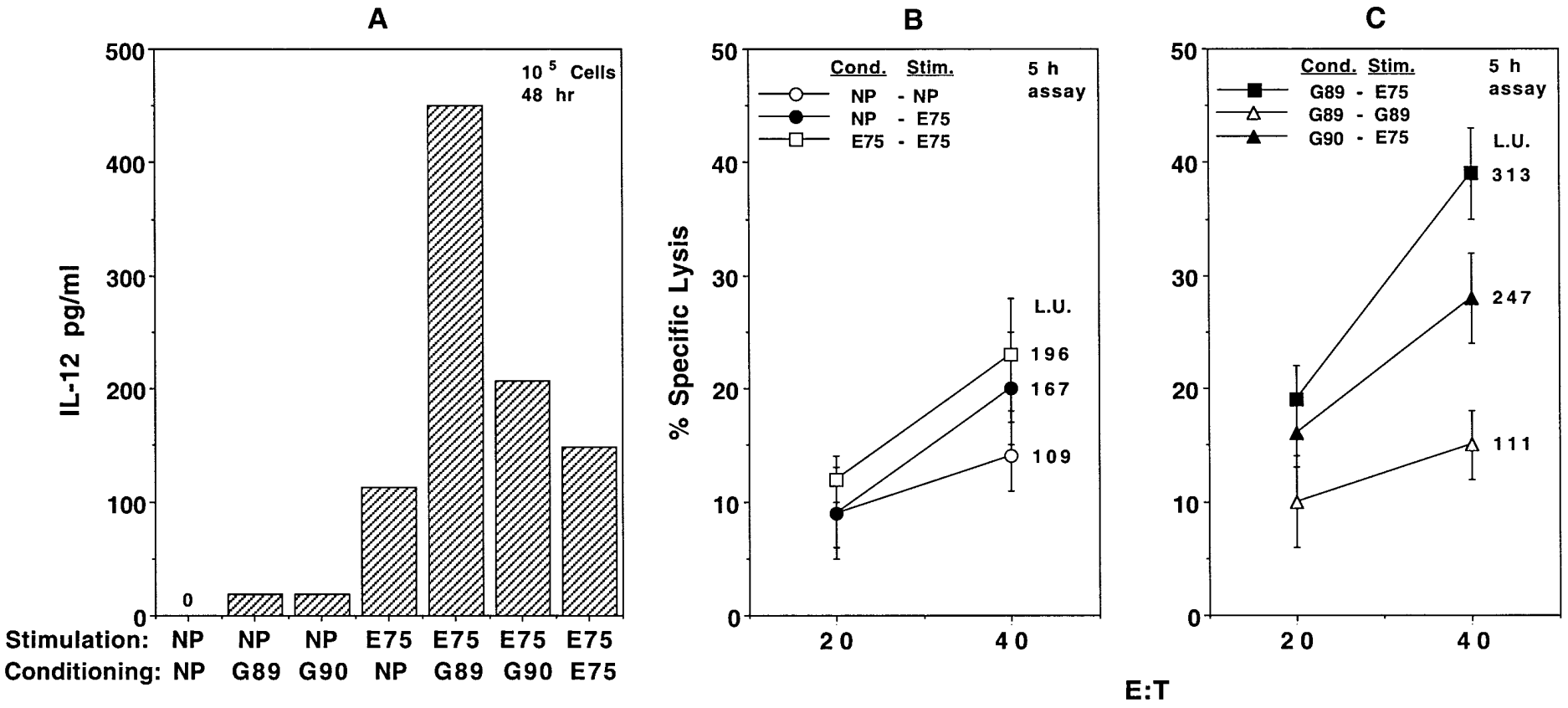

A
B
C
IL-12 pg/ml
500
400
300
200
100
0
10 5 Cells
48 hr
0
Stimulation: NP NP NP E75 E75 E75 E75
Conditioning: NP G89 G90 NP G89 G90 E75
% Specific Lysis
50
40
30
20
10
0
Cond. Stim.
5 h assay
NP - NP
NP - E75
E75 - E75
L.U.
196
167
109
20
40
E:T
G89 - E75
G89 - G89
G90 - E75
313
247
111

FIGURE 3

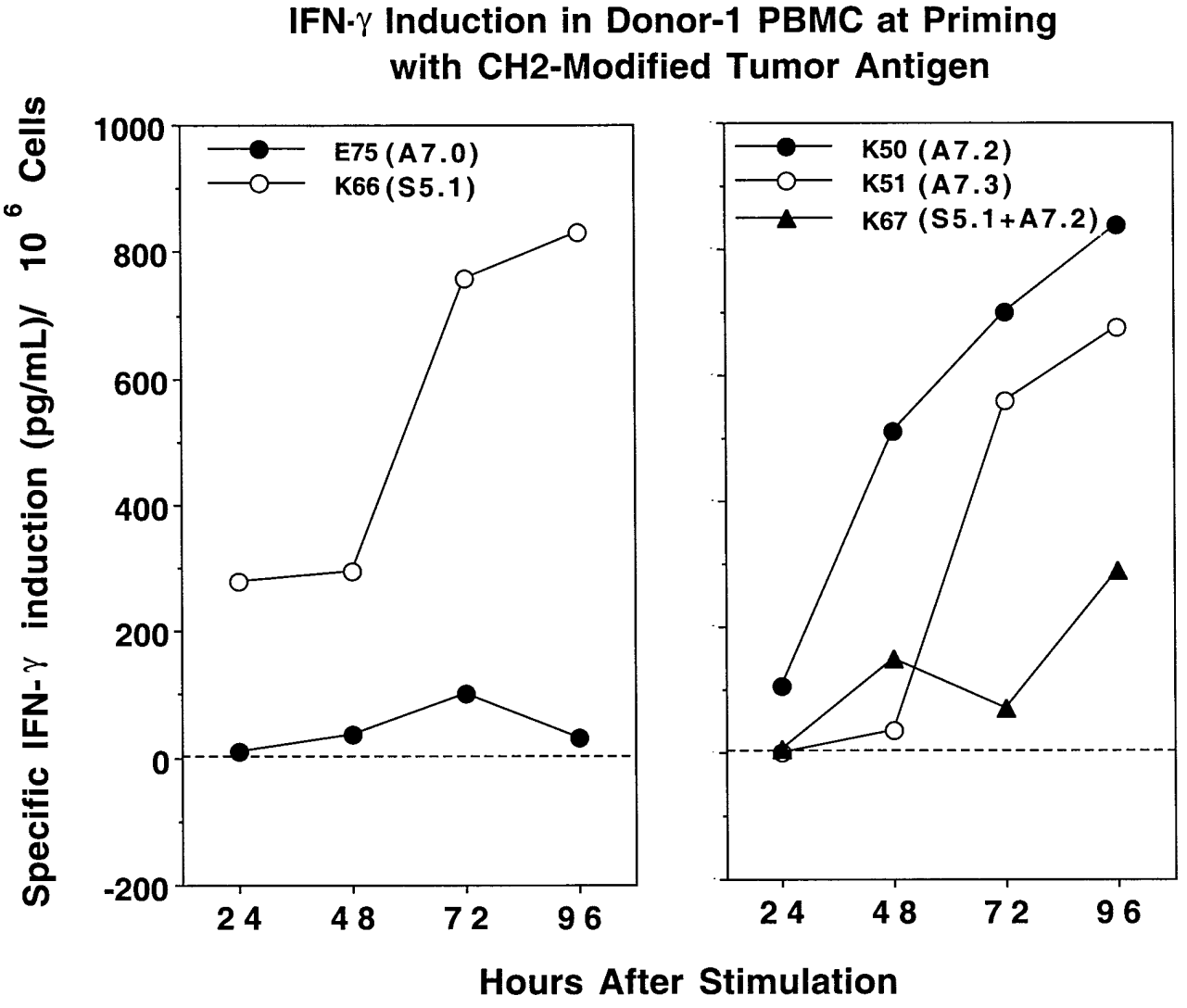

IFN-γ Induction in Donor-1 PBMC at Priming with CH2-Modified Tumor Antigen
Specific IFN-γ induction (pg/mL)/ 10 6 Cells
1000
800
600
400
200
0
-200
E75 (A7.0)
K66 (S5.1)
K50 (A7.2)
K51 (A7.3)
K67 (S5.1+A7.2)
24
48
72
96
Hours After Stimulation

FIGURE 4

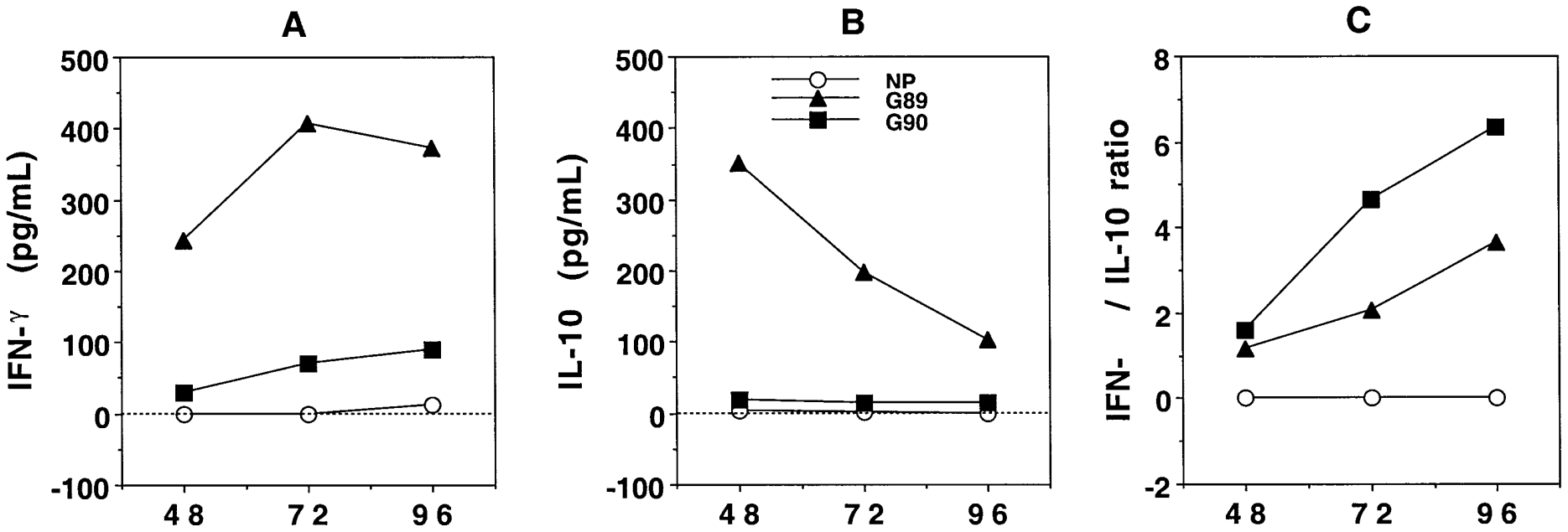
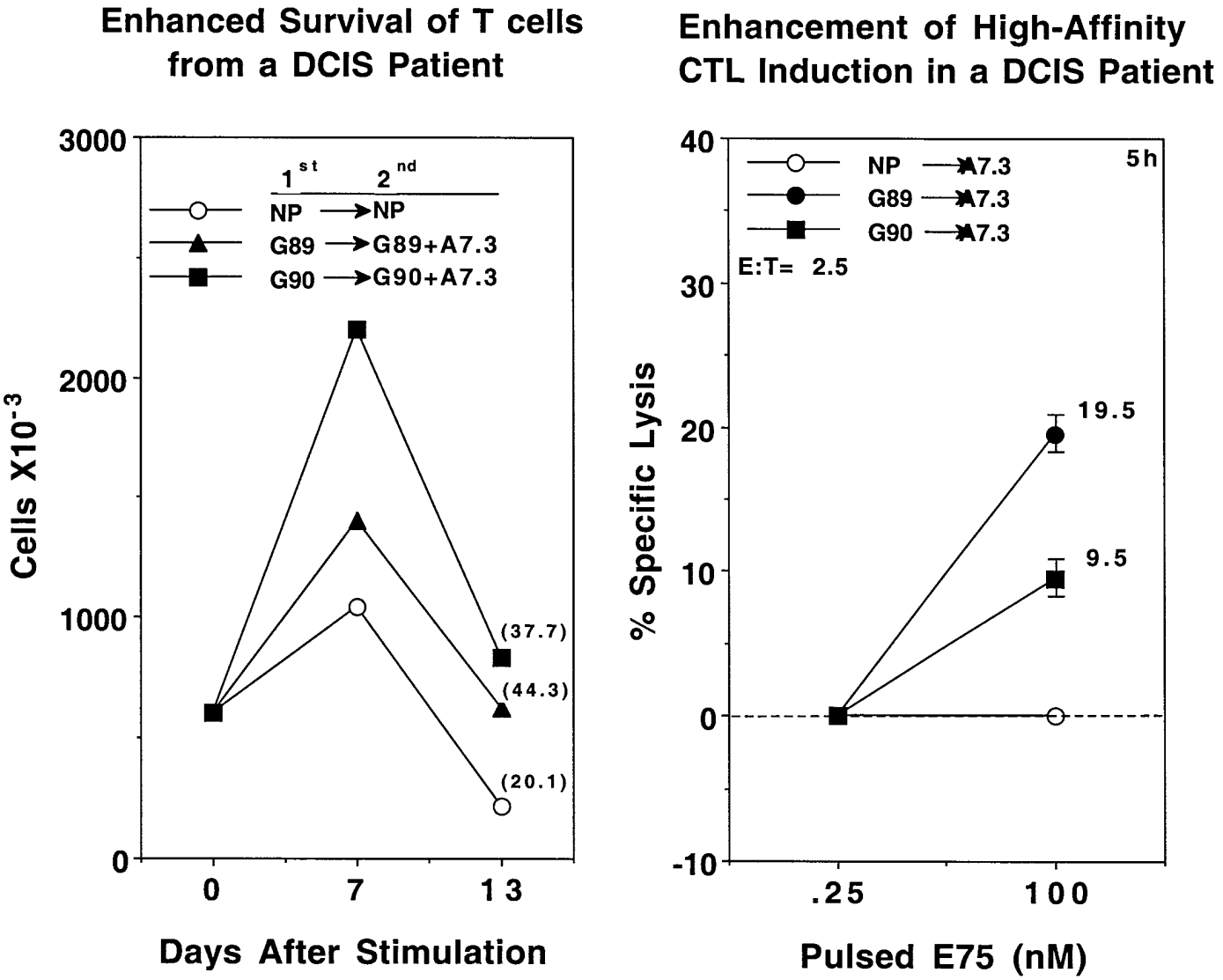

A
B
C
IFN-γ (pg/mL)
IL-10 (pg/mL)
IFN- / IL-10 ratio
500
400
300
200
100
0
-100
8
6
4
2
0
-2
48
72
96
NP
G89
G90

FIGURE 5


Enhanced Survival of T cells from a DCIS Patient
3000
2000
1000
0
Cells X10-3
1st 2nd
NP →NP
G89 →G89+A7.3
G90 →G90+A7.3
(37.7)
(44.3)
(20.1)
0
7
13
Days After Stimulation
Enhancement of High-Affinity CTL Induction in a DCIS Patient
40
30
20
10
0
-10
% Specific Lysis
NP →A7.3
G89 →A7.3
G90 →A7.3
5h
E:T= 2.5
19.5
9.5
.25
100
Pulsed E75 (nM)

Peptide Priming of Cytolytic Activity to HER-2 Epitope 369–377 in Healthy Individuals[1]

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ABSTRACT

The presence of tumor-reactive CTLs in tumor infiltrates and in the peripheral blood of cancer patients demonstrates an immune response against tumors that apparently cannot control disease spread. This raises concerns as to whether amplification of this response may be useful during disease progression. Induction of tumor-reactive CTLs in healthy donors at risk, as well as in patients free of disease, may be therapeutically important, based on the hypothesis that CTLs that recognize tumors early may be more effective in containing their progression than CTLs that expand only when the disease progresses. To address the feasibility of priming cytolytic activity in healthy donors, we used the HER-2 peptide E75 (369–377) as an immunogen and autologous peripheral blood mononuclear cell-derived dendritic cells as antigen-presenting cells. We found that of 10 healthy donors tested, two responded at priming with E75 presented on autologous dendritic cells by induction of E75-specific CTL activity. Three other responders were identified after two additional restimulations. Of these five responders, three recognized E75 presented on the ovarian tumor line SKOV3.A2, as demonstrated by cold-target inhibition experiments. Induction of cytolytic activity at priming was enhanced in responders by tumor necrosis factor-α and interleukin 12 but not in the nonresponders. αB7.1 monoclonal antibody added at priming enhanced induction of lytic activity in only one of the four nonresponding donors tested, suggesting that in the majority of donors, E75-precursor CTLs were not tolerized. Because of the possibility that disease may develop in nonresponders, strategies to improve the immunogenicity of tumor antigens for healthy donors may be required for development of cancer vaccines.

INTRODUCTION

Identification of human epithelial tumor Ags,[4] such as the ones expressed on ovarian and breast cancers, allows antitumor vaccination strategies to be developed. Among the most interesting are those that focus on *HER-2* because this proto-oncogene is overexpressed in 20–40% of patients with highly aggressive breast, ovarian, pancreas, colon, and prostate cancers and with consequent poor prognosis. Two clinical trials have targeted *HER-2* (1, 2) using peptides and various adjuvants. The immunogen of choice in these trials was of the HER-2 peptide E75 (369–377, KIFGSLAFL), which maps an epitope frequently recognized by CTLs from tumor-infiltrating/associated lymphocytes of breast and ovarian cancer patients (3, 4).

Although peptide immunization is an appealing approach to tumor immunotherapy because it removes concerns of toxicity and safety while focusing the effectors, the methodology for vaccination and immunological evaluation it is not yet defined (5). Important questions need to be addressed before this approach can be developed to its therapeutic potential. The first question is whether CTLs generated by primary *in vitro* and *in vivo* immunizations will lyse targets endogenously expressing the Ag. It has been shown with model Ag that the majority of peptide-induced CTLs at priming recognized the peptide used as immunogen, but only a small fraction recognized the endogenously presented Ag (6). In some instances, CTLs recognizing endogenously presented Ag could be induced only with a variant peptide (7). Although peptide-specific $CD8^+$ cells may not always be expected to directly lyse tumors *in vitro* and *in vivo*, such cells can recognize peptides derived from extracellular degradation of Ag from dying tumor cells and tumor debris. This may lead to secretion of various cytokine patterns in the tumor environment and conditioning of APCs, resulting in an indirect but significant impact on antitumor responses.

The second concern is how frequently tumor peptide vaccinations induce Ag-specific CTLs in the human population. This concern is attributable to the reported low precursor frequency of tumor-reactive CTLs in healthy individuals, however, this concept has been recently challenged (8, 9). There is also concern over the weak ability of tumor Ag to induce massive Ag-specific CTL expansions, as reported with viral Ags (10).

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[4] The abbreviations used are: Ag, antigen; DC, dendritic cell; *HER-2*, *HER-2/neu* proto-oncogene; HS, human serum; NP, no peptide; APC, antigen-presenting cell; PBMC, peripheral blood mononuclear cell; GM-CSF, granulocyte/macrophage-colony stimulating factor; IL, interleukin; mAb, monoclonal antibody; TNF, tumor necrosis factor; LU, lytic unit(s); rVV, recombinant vaccinia vector.

The third concern is whether self-reactive (tumor-reactive) CTLs in healthy donors are silenced by active tolerance or anergy, and stimulation with Ag in peptide form cannot reactivate memory effectors because of B7-CTLA4-mediated peripheral tolerance. Recent studies have shown that induction of melanoma tumor Ag and tumor-reactive CTLs in healthy donors is much less effective than in cancer patients (10). However, induction of tumor-reactive CTLs in healthy donors (as well as in breast and ovarian cancer patients in long-term remission and without evidence of disease) is important based on the hypothesis that CTLs that recognize tumors early may be more effective in containing their progression than CTLs that expand only when the tumor Ag is overexpressed. (11).

Although a number of studies focused on improving the immunogenicity of tumor peptides in selected responding patients and donors using DCs as APCs and inflammatory cytokines, there is little information on the frequency of induction of these responses in unselected healthy donors. However, this question is important because cancer vaccines are expected to be given to distinct individuals, of which some may be at risk to develop disease, whereas others may be free of disease and otherwise considered healthy individuals. Thus, the frequency of CTL responses to a tumor Ag in the population becomes an important issue. We rationalized that if the frequency of responses to E75 priming is similar to or lower than the frequency of responses to MART-1 (10), initial screening of a large panel of healthy donors may identify at least one responder. Cells of this responder can be then used as positive controls to address the questions of costimulation and of cytokine help in elicitation of cytolytic function in nonresponders.

We developed a model for priming T cells of PBMCs from healthy donors with the HER-2 peptide E75. We used as APCs autologous DCs, always freshly generated in the presence of GM-CSF + IL-4 from the same PBMC sample. To determine the role of costimulation in this system, αB7.1 antibodies were added at priming. To establish whether IL-12 and TNF-α are essential for CTL priming, stimulations were performed in the presence or absence of these cytokines. We found that 2 of 10 healthy donors responded by inducing E75-specific cytolysis at peptide priming and 5 of 10 at restimulation. Although IL-12 and TFN-α potentiated CTL induction in the responsive donors, they did not help induce CTLs in nonresponders, suggesting that additional factors to the nature of APCs and inflammatory cytokine conditioning regulate the induction of CTLs specific for *HER-2* by synthetic peptides.

MATERIALS AND METHODS

Cells, Antibodies and Cytokines. HLA-A2$^+$ PBMCs were obtained from healthy volunteers from the Blood Bank of M. D. Anderson Cancer Center. The HLA phenotypes of the donors used in this study were: donor 1 (A2, B7, 44); donor 2 (A2, 33, B40, 44); donor 3 (A2, 33, B41, 81); donor 4 (A1, 2, B27, 44); donor 5 (A1, 2 B44, 57, Cw5, 6); and donor 6 (A2, 31, B35, 44, Cw4, w5). For the other four donors only, the HLA-A2 expression was determined. T2 cells, ovarian SKOV3, SKOV3.A2 cells, and indicator tumors from ovarian ascites were described (3, 4). mAb to CD3, CD4, CD8 (Ortho), CD13 and CD14 (Caltag Laboratories, San Francisco, CA), B7.1 and B7.2 (CD80 and CD86, Calbiochem), intercellular adhesion molecule-1 (ICAM-1 CD54; Calbiochem), CD40L (Ancell, Bayport, MN), HLA-A2 (clone BB7.2; American Type Culture Collection), and MHC-II (L243; Dako Corp., Carpinteria, CA) were used as unconjugated, FITC, or phycoerythrin conjugated. The following cytokines were used: GM-CSF (Immunex Corp., Washington, DC; specific activity, 1.25×10^7 colony-forming units/250 mg); TNF-a (Cetus Corp., Emeryville, CA; specific activity, 2.25×10^7 units/mg); IL-4 (Biosource International; specific activity, 2×10^6 units/mg); and IL-2 (Cetus Corp.; specific activity, 18×10^6 IU/mg). IL-12 at 5×10^6 units/mg was a kind gift from Dr. Stanley Wolf (Department of Immunology, Genetics Institute, Cambridge, MA).

Synthetic Peptides. The HER-2 peptides used were: E75 (369–377) and the unnatural modified Muc-1 peptides D125: (GVTSAKDTRV) and D132 (SLADPAHGV). The corresponding natural peptides do not bind HLA-A2. Introduction of an HLA-A2 anchor and sequence modification in Muc1 in residues contacting TCR lead to an unnatural sequence (12). All peptides were prepared by the Synthetic Antigen Laboratory of M. D. Anderson Cancer Center and purified by high-performance liquid chromatography. Peptides were 95–97% pure by amino acid analysis. Peptides were dissolved in PBS and stored frozen at −20°C in aliquots of 2 mg/ml Polyglycol bead-containing E75 were a kind gift of Dr. Kenneth Grabstein (Corixa Corp.).

Immunofluorescence. Antigen expression by DCs and T cells was determined by fluorescence-activated cell sorter using a flow cytometer (EPICS Profile Analyzer; Coulter Co., Hialeah, FL). DCs were defined by the presence of CD13 and absence of CD14 marker after culture in GM-CSF and IL-4. For phenotype analysis, DCs were incubated with phycoerythrin-conjugated anti-CD13 mAb and FITC-conjugated mAb specific for a surface Ag.

Generation of PBMC-derived DCs. CD13$^+$ DCs were generated from freshly isolated PBMCs by following the established CD14 methods (13, 14). Complete RPMI 1640 (containing 10% FCS) supplemented with 1000 units/ml GM-CSF, and 500 units/ml IL-4 were added to each well containing plastic-adherent cells, changed every 48 h, and maintained for 7 days. In separate studies, performed in parallel, we attempted to grow DCs in medium containing either HS or in AIM-V medium. The growth and expression of surface markers of DC cultured in complete RPMI 1640 was significantly better than of DCs cultured in other conditions; thus, in this study only DCs cultured in complete RPMI 1640 were used. CD8$^+$ cells were isolated by removing first the CD4$^+$ and then the CD16$^+$ and CD56$^+$ cells from the nonadherent population using Dynabeads (Dynal, Oslo, Norway). After depletion, the resulting cells were 97% CD8$^+$, as determined by flow cytometry.

T-Cell Stimulation by Peptide-pulsed DCs. DCs were washed three times in serum-free medium, plated at 1.2×10^5 cell/well in 24-well culture plates, and pulsed with peptides at 25–50 μg/ml in serum-free medium for 4 h before the addition of responders. TNF-α (50 units/ml) was added to DCs for the last hour to stimulate Ag uptake and presentation (13). Autologous PBMCs or isolated CD8$^+$ cells in RPMI 1640 containing 10% HS were added to DCs at 1.5×10^6/ml, followed 60 min later by IL-12 at 3 IU/ml, IL-2 was added 16 h later to each well at 60 IU/ml and every 48 h thereafter. For inhibition studies,

Table 1 Summary of E75-specific cytolytic responses in healthy donors

Donor no.	E75-specific response at primary	Enhancement by IL-12/ TNF-α	E75-specific CTL at restimulation[a]	Specific tumor lysis	No. of independent priming experiments
1	+	+	+ (1)[b]	+	6
2	−	−	− (2)	ND	3
3	−	−	+[c] (3)	+	6
4	−	−	− (3)	ND	4
5	+	+	+ (1)	+	4
6	−	−	+[c] (3)	−	5
7	±[d]	±	+ (1)	−	4
8	−	−	− (2)	ND	4
9	−[e]	−	− (2)	ND	3
10	−	−	− (1)	ND	1
Total	2/10	2/10	5/10	3/5	40

[a] +, specific lysis of E75-pulsed T2 was ≥20% at an E:T ratio of 20:1; −, specific lysis of E75-pulsed T2 was not significantly different than lysis of control T2 cells pulsed with no peptide. ND, not done.

[b] Numbers in parentheses, number of restimulations.

[c] E75-specific CTL activity was detected after four stimulations.

[d] E75-specific CTL activity was ≤10% at an E:T of 10:1.

[e] E75-specific activity, observed occasionally, could not be enhanced by peptide plus cytokine stimulation but was enhanced by αB7.1.

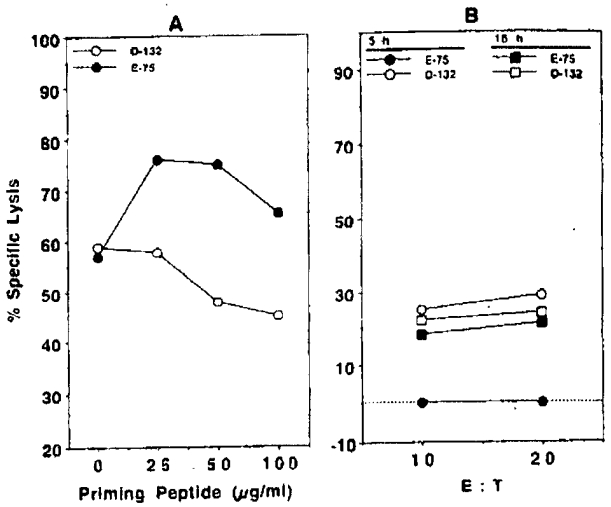

Fig. 1 *A*, induction of E75-specific cytolytic activity at priming with various concentrations of E75. Plastic nonadherent PBMCs from donor 1 were stimulated with the indicated concentrations of E75. Seven days later, cells were removed from cultures and tested for CTL activity against T2 pulsed with 25 μg/ml E75 or D132. The experiment was performed in triplicate at a 20:1 ratio from all stimulation groups. Differences between T2-E75 (●) recognition and T2-D132 recognition (○) are significant ($P < 0.05$) in all DC-E75 stimulation groups. *B*, priming of PBMCs from donor 9 (nonresponder) with E75 in polyglycol beads does not induce E75-specific CTL activity. Experimental conditions were as described for donor 1.

mAbs specific for B7.1, B7.2, and HLA-A2 were added to DCs 1 h before responders in amounts reported to be inhibitory by the manufacturers.

CTL and Cytokine Assays. Recognition of peptides used as immunogens by CTLs was performed as described (3, 15). Equal numbers of viable effectors from each well were used in all assays. To minimize cross-reactive recognition of human peptides, all stimulations were preformed in medium containing HS, whereas CTL assays were performed using medium containing FCS (15). This minimized background cytolysis attributable to activation of other autoreactive cells to Ags present in HS. Specific LU were determined as described (10, 16) and are expressed as LU $30/10^7$ cells.

RESULTS

Priming of Healthy Donors PBMCs with E75-pulsed Autologous CD14-derived DCs. To address the question of whether priming with E75 presented on DCs induce E75-specific CTL activity, plastic nonadherent PBMCs from healthy donors were stimulated with autologous DCs generated by culture in GM-CSF + IL-4. DCs were pulsed with E75 at 25–50 μg/ml. Seven days later, cytolytic activity was determined against E75-pulsed T2 using as control T2 cells that were not pulsed with peptide. Cumulative results are presented in Table 1. The results show that of 10 healthy donors tested, only 2 showed stable and consistent recognition of E75 after priming with DC-E75. These cumulative results show that E75 can induce specific cytolytic activity at priming only in a small fraction of healthy donors (2 of 10; 20%). These results were confirmed because each CTL induction experiment was repeated at least three times at different time points and always with freshly isolated PBMCs (except donor 10). In 2 donors (donors 2 and 9), specific CTL activity was occasionally detected in one of three independent experiments, but this activity was unstable and could not be further expanded. Induction of this activity in donor 9 required αB7.1 at priming (shown in Fig. 5). In contrast, in the two responding donors, specific CTL activity was detected at priming in 4 of 6 (donor 1) and 3 of 4 (donor 5) independently performed induction experiments over periods of 1 year and 6 months, respectively.

To address the question of the optimal Ag concentration for priming, autologous DCs were pulsed with 0, 25, 50, and 100 μg/ml of E75 prior to the addition of responders. The resulting cultures were supplemented with TNF-α and IL-12 at priming and IL-2 every 48 h for the following 7 days. A representative experiment of the specificity of recognition of CTLs from donor 1 primed by DC-E75 is shown in Fig. 1. The results show that the specificity of recognition of CTLs induced by E75 at three concentrations of peptide (25, 50, and 100 μg/ml) was similar, with 50 μg/ml E75 inducing the highest differences in lytic activity between targets pulsed with E75 or targets pulsed with no peptide. CTLs could not be induced even by high concentrations of E75 in any of the nonresponders. To address whether E75-stimulatory potency was increased by delivery of Ag encapsulated in polyglycol beads, PBMCs from two nonresponders (numbers 2 and 9) were stimulated with autologous DCs pulsed with polyglycol beads in numbers to generate equivalent E75 concentrations with peptides. The results are shown for donor 9 (Fig. 1*B*). Stimulation with encapsulated-E75 failed to induce specific CTL activity at priming, as concluded from 5-h CTL assays. However, in longer CTL assays (16 h), lysis of T2-E75 pulsed targets increased significantly compared with

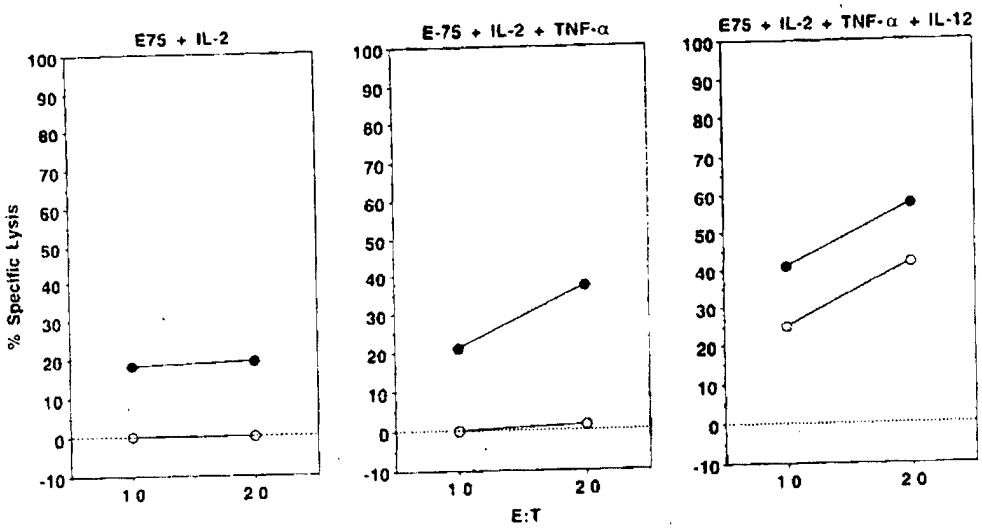

Fig. 2 Induction of E75-specific cytolytic activity at priming in donor 5. Plastic non-adherent PBMCs were stimulated with 50 μg/ml E75 pulsed on autologous DCs. TNF-α (50 IU/ml), IL-12 (3 IU/ml), and IL-2 (60 IU/ml) were added to these cultures. Differences in lysis of T2 cells pulsed with E75 (●) or not (○) are significant in all groups ($P < 0.02$). All experiments were performed simultaneously in triplicate. Results of one experiment of two performed with similar results are shown.

that of control targets, and the levels of recognition were similar. This suggested that either the numbers of E75-specific CTLs were low, or CTLs in these two donors had weak cytolytic activity. It should be mentioned that in both experiments, IL-2 was added at 48 and 96 h in higher concentrations (150–200 IU/ml) to facilitate T-cell expansion. Although this increased the background lysis, it did not change the patterns of recognition.

Effects of TNF-α and IL-12 in Induction of CTL Activity at Priming. Both TNF-α and IL-12 have been described in different systems to augment cytotoxicity of $CD8^+$ cells (17, 18). To determine whether the E75-induced CTL activity could be enhanced by TNF-α and IL-12, CTL priming experiments were performed in the presence and absence of these cytokines (Fig. 2). The results from donor 5 show that the addition of TNF-α at the time of pulsing with peptide-enhanced, E75-specific activity compared with cultures that received only IL-2. When IL-12 was added, the increase in T2-E75 killing was paralleled by an increase in nonspecific killing. The addition of IL-12 at higher concentrations during priming did not increase the specific but rather the nonspecific CTL activity. This was equally true when isolated $CD8^+$ cells were used as effectors (data not shown). To address whether these cytokines enhanced cytolytic activity at priming in nonresponders, the experiment was repeated with donor 1 (responder) and donor 4 (nonresponder). The results in Fig. 3 show that TNF-α increased specific CTL activity in donor 1 but not in donor 4. Although T2-E75 lysis increased in TNF-α-treated cultures, it was still not significantly different from the control. Similar to donor 5 (in both donors 1 and 4), IL-12 increased both the nonspecific and specific lysis. These results were confirmed with all donors tested (Table 1). IL-12 alone, or together with IL-2 and TNF-α, failed to induce specific CTL activity in nonresponders.

To address whether the increase in cytolytic activity induced by TNF-α and IL-12 at priming was attributable to changes in the levels of $CD8^+$ cells in these cultures, we determined the percentages of $CD8^+$ and $CD4^+$ in E75-primed cultures from donor 5 used in the experiment shown in Fig. 2. The results in Table 2 show only small differences between the

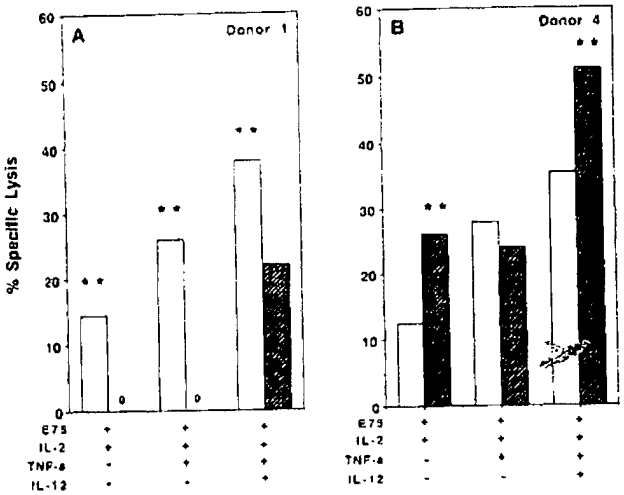

Fig. 3 Addition of IL-12 at priming does not induce specific CTL activity in the nonresponders. Donor 1 (responder; *A*) and donor 4 (nonresponder; *B*) were tested in the same experiment. The E:T ratio was 20:1. For donor 1, this experiment was performed at a different date with a fresh blood sample than the experiment shown in Fig. 1. *A*: **, significant differences in recognition of E75 (□) compared with control NP (■; $P < 0.02$). *B*: **, significantly higher recognition of T2-NP than of T2-E75 ($P < 0.05$).

percentage of $CD8^+$ cells in cultures containing IL-2 only compared with IL-2 + TNF-α or IL-2 + TNF-α + IL-12. A caveat of this analysis is that E75 tetramers are not yet available; thus, we could not determine whether TNF-α and IL-12 increased the numbers of E75-specific cells in these cultures.

Involvement of B7-CD28 Costimulation in E75-specific CTL Priming. To address whether induction of E75-specific cytolytic activity required costimulation, αB7.1 and αB7.2 mAbs were added at priming. The results in Fig. 4 show that αB7.1 significantly inhibited induction of specific CTL activity in donor 5 (by >80%), whereas αB7.2 had a much smaller effect.

Table 2 Expression of CD8 and CD4 on E75-primed cultures

Peptide	IL-2	TNF-α	IL-12	%CD8⁺	%CD4
–	+	–	–	21.3	59.1
+	+	–	–	18.9	64.9
–	+	+	–	N.D.	N.D.
+	+	+	–	21.4	63.4
–	+	+	+	25.2	55.9
+	+	+	+	25.1	55.0

Plastic-nonadherent PBMCs from donor 5 were stimulated with autologous DCs pulsed with E75, or as control with autologous DCs that were not pulsed with E75. TNF-α and IL-12 were added at priming, whereas IL-2 was added 24 h later. Cells were stained with the corresponding α-CD8 and α-CD4 in antibody 7 days later and examined by fluorescence-activated cell sorter.

B7.2 was expressed at significantly higher levels than B7.1 on DCs. Furthermore, interaction of DCs with T cells and cytokines was paralleled by B7.2 but not B7.1 up-regulation within 20 h (not shown), raising the possibility that αB7.2 mAb was insufficient for blocking. The strong inhibition of E75-specific CTL induction by αB7.1 suggested that the responder CTLs in this donor are more likely naïve T cells.

To determine whether the nonresponders were activated but tolerized T cells, which cannot expand because of B7-CTLA4 interaction, the experiment was repeated with four nonresponders (nos. 3- 6) using the same amounts of αB7.1 as in donor 5. The results are shown in Fig. 5. In donor 9, the addition of αB7.1 at priming led to induction of specific CTL activity (Fig. 5*A*). It is tempting to speculate that in this donor, activated but tolerized E75-specific CTLs were present, and they cannot expand because of the B7-CTLA4 interaction. Additional studies are needed to address this point. In donors 4 and 5, the addition of αB7.1 at priming failed to induce significant specific cytolytic activity. These results were confirmed with donor 3 (not shown). Thus, of five donors tested, αB7.1 inhibited E75-specific CTL priming in one (no. 5), enhanced CTL priming in another one (no. 9), but failed to enhance induction of specific CTL activity in three (nos. 3–5). These experiments were repeated, and the results were confirmed. Thus, the requirements for B7-CD28 costimulation appeared to be dependent on the donor.

Induction of CTL Activity at Restimulation. To address whether E75 restimulation enhanced specific cytolytic activity, E75 primed PBMCs from all donors were restimulated with DC-E75. Of the eight nonresponders at primary stimulation, only three (donor nos. 3, 6, and 7) increased their E75-specific lysis at restimulation. In two of three donors (donors 3 and 6), specific E75 recognition was borderline after restimulation. E75-specific cytolysis was observed at the fourth stimulation with DC-E75 in these two donors (data not shown). In the other five donors that were both primed and restimulated with E75 but failed to show specific CTL activity, additional restimulations were not attempted, because of the low levels of recognition of T2-E75 at restimulation compared with nonspecific lysis. We rationalized that the five nonresponders will require a minimum of four and even more restimulations to possibly elicit E75-specific CTLs. Thus, even if cytolytic activity would be detected after four to five stimulations, this finding would also support the hypothesis of weak E75 immunogenicity in these individuals.

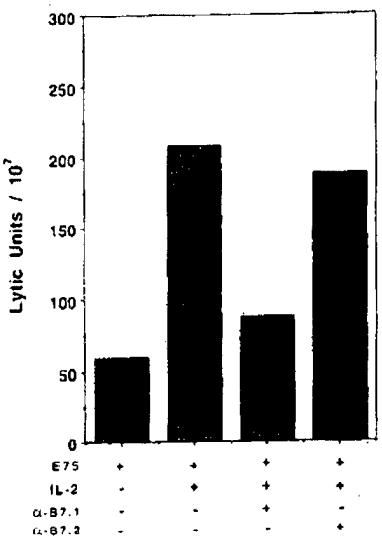

Fig. 4 Priming of E75-specific cytolysis in donor 5 requires B7 costimulation. Plastic nonadherent PBMCs from donor 5 were primed with E75 in the presence or absence of αB7.1 and αB7.2 mAb. Results are shown as specific LU calculated from two E:T ratios (10:1 and 20:1) from the percentage of specific lysis against E75 and against the specificity control, the unnatural negative control peptide D132 (10, 12). D132 was used as control to stabilize HLA-A2 on T2 cells similarly with E75.

Recognition of Tumor Cells by E75-primed CTLs. To address the question of whether E75-primed CTLs from healthy donors recognized endogenously presented epitopes, CTLs generated from donors 1, 3, 5, 6, and 7, which showed peptide-specific lytic activity, were tested for their ability to lyse the tumor line SKOV3.A2 and its A2⁻ counterpart, SKOV3. Except for HLA-A2, all other histocompatibility Ags on SKOV3 and SKOV3.A2 are identical. To verify that the responses are E75 specific, cold-target inhibition experiments using unlabeled T2-E75 as specific target and T2-NP as negative control were performed in parallel. T2 express only HLA-A2 and low levels of HLA-B5. The results are summarized in Table 1. Peptide-specific CTLs from donors 6 and 7 did not show specific recognition of SKOV3.A2 tumor. However, E75-specific CTLs from donors 1, 3, and 5 recognized endogenous E75. These donors do not express HLA-B5, suggesting that E75 was presented by HLA-A2. It should be mentioned that E75-specific CTLs were induced in donors 1 and 5 at priming with DC-E75, whereas in donor 3, expression of this cytolytic activity required four stimulations with DC-E75. These results indicated that of 10 healthy donors tested, only three (33%) responded by inducing CTLs that specifically recognized tumor cells. This percentage was higher in the group of responders with peptide-specific CTLs (three of five; 60%). The results with donor 5 are shown in Fig. 6, *A* and *B*. Both E75-primed cultures from donor 5 lysed SKOV3.A2 better than SKOV3, suggesting that they recognize an epitope associated with HLA-A2. To address whether these cultures recognized an endogenous presented epitope similar to E75, we performed cold-target inhibition experiments. The results in Fig. 6*C* show that T2-E75 significantly inhibited by >50% recognition of SKOV3.A2 by CTLs from donor 5 com-

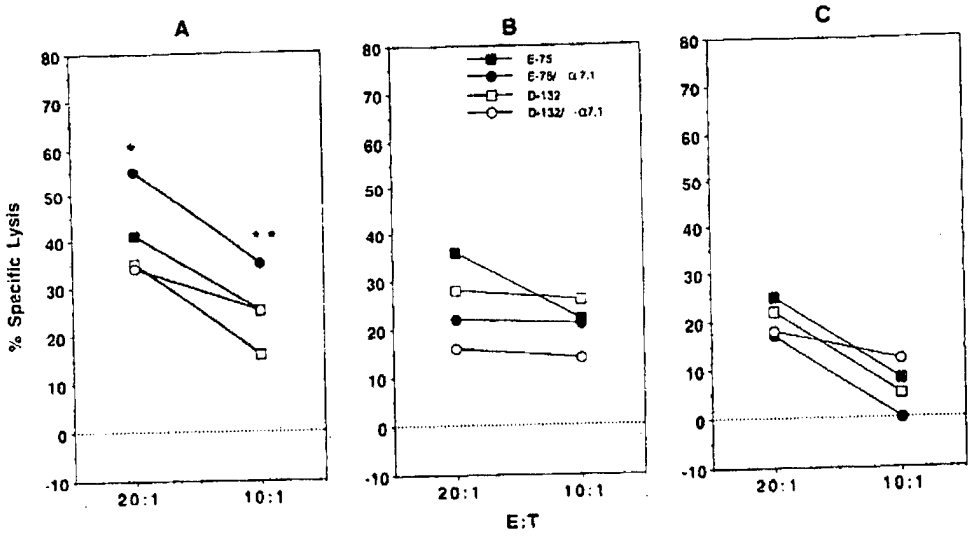

Fig. 5 Effects of B7.1 blocking on priming of E75-peptide-specific cytolytic activity in distinct healthy donors. Induction of E75-specific cytolysis by blocking of B7.1 in donor 9 (*A*) but not in donors 3 and 4 (*B* and *C*). αB7.1 was added to DCs 30 min before the addition of responders. Cultures were primed with E75 in the presence (●) or absence (□, ■) of αB7.1 in the same experiment. Cytolytic activity was determined in the same experiment in triplicate against targets pulsed with E75 (■, ●) or the control unnatural peptide D132 (□, ○). D132 was used to stabilize HLA-A2 at comparable levels with E75, thus minimizing the natural killer-derived background lysis. * and **, $P < 0.01$ and <0.05 respectively, compared with the lysis of T2-D132.

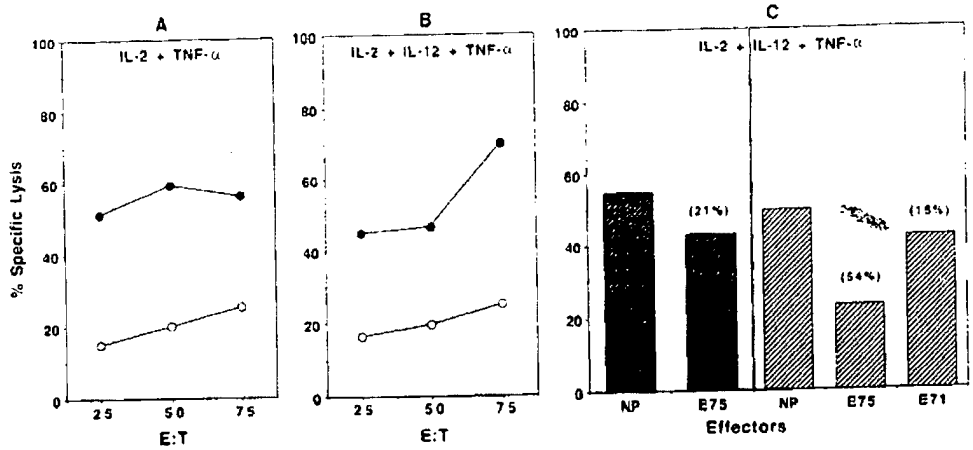

Fig. 6 *A* and *B*, E75-primed PBMCs from donor 5 recognize SKOV3.A2 (●) better than SKOV3 (○) cells, suggesting that they recognize an HLA-A2-associated Ag. Differences between the levels of lysis of two targets are significant ($P < 0.05$). *C*, cold-targeted inhibition of SKOV3.A2 lysis by E75 + IL-2 + TNF-α-induced cells by T2-E75, but not by negative control T2-NP, or the negative peptide control T2-E71 demonstrate E75-specific recognition for donor 5 (■) and donor 1 (□). Differences in lysis of SKOV3.A2 cells incubated with T2-NP (percentage of specific lysis: 56, 59, and 60%) and T2-E75 (percentage of specific lysis: 39, 42, and 44%) were significant ($P < 0.05$).

pared with control T2-NP, which expressed HLA-A2. This inhibition was peptide specific because it was not observed with the control peptide E71 pulsed on T2, suggesting that some E75-primed CTLs recognized an endogenously presented epitope, but these cells are not the majority in the effector population. Similar results were obtained with E75-primed cells from donors 1 (Fig. 6*C*) and 3 (not shown). However, the levels of cold-target inhibition were lower and ranged between 20 and 25% in two separate experiments. This suggested that only a subpopulation of E75-induced CTLs recognize endogenous generated epitopes. Thus, successful induction of E75-specific CTL activity at priming with E75 using DCs as APCs and inflammatory cytokine support appears to be dependent of additional factors other than the nature of APCs and B7 costimulation.

DISCUSSION

In this study, we investigated the ability of the HER-2 peptide E75 to prime E75-specific cytolytic activity in healthy donors when presented on autologous DCs. We found that only

2 of the 10 HLA-A2$^+$ healthy donors tested responded by induction of E75-specific cytolytic activity at priming. This was confirmed in replicated experiments performed over time, and the use of various cytokine combinations IL-2+IL-12, IL-2 + TNF-α + IL-12, or preculture in IL-2, preculture in IL-2 + RANTES.[5] These results indicated that E75-specific or cross-reactive T cells endowed with cytolytic activity can be elicited at priming in only a fraction of healthy donors (20%) but induced in an additional 30%. Of interest, E75-primed CTLs from these two donors recognized E75 presented on tumor cells, because their activity was inhibited in cold-target inhibition assays.

Two cytokines, TNF-α and IL-12, were used to potentiate E75-specific CTL induction. TNF-α has been described to increase Ag uptake and presentation by DCs (13) and to potentiate CTL generation in animal models (17). IL-12 has also been described to potentiate CTL induction and cytolytic activity (18, 19). TNF-α and IL-12 increased the levels of cytolytic activity in responders but had no effect in nonresponders. This suggests that these cytokines are not essential for priming of E75-specific CTL activity. Of interest, in the responders, induction of E75-specific cytolytic activity was inhibited by αB7.1, suggesting a requirement for costimulation in the induction of cytolytic activity, raising the possibility of DC-E75 primed naïve T cells.

Induction of E75-specific cytolytic activity at priming with peptide observed with two healthy donors is of interest in evaluating the potential of this epitope for tumor-specific CTL induction and cancer vaccine development. In human tumor systems (most instances), priming with peptide required several repeated stimulations of healthy donor PBMCs before specific cytolysis was detected. CTLs specific for tyrosinase 369–377 peptide were detected in four of five healthy donors after three restimulations with peptide (21). Peptide-specific CTLs were induced in healthy donors using DCs and peptides from gp100, tyrosinase, and MART-1/MelanA. Detection of CTL activity required three to four cycles of stimulation (22). Similarly, presentation of MART-1 by DCs transduced with an adenoviral vector construct carrying the *MART-1* gene required three stimulations for induction of specific cytolysis (23), although in some donors, specific cytolytic effectors were detectable 7 days after priming (24). In contrast, in another study, MART-1 (27–35)-specific cytolytic function could be induced in a nonresponder only, using APCs infected with rVVs expressing rVV-B71/2 + peptide, or rVV-B7.1 + MART-1 and restimulated with peptide, but not by peptide stimulation only (25, 26). Thus, the potency of E75 to induce cytolytic function in healthy donors appears similar to that of the MART-1 peptide 27–35.

Few studies have investigated the frequency of responses to tumor Ags in healthy donors at priming and restimulation or the consistency of these responses for an individual. This aspect is important because of its implications for protective vaccination in healthy donors or ovarian, breast, and prostate cancer patients without evidence of disease. In one extensive study, Marincola *et al.* (10) found that after several stimulations with MART-1 (27–35), five of nine healthy donors (56%) responded by induction of specific cytolytic effectors. Only one to two donors showed weak activation of cytolysis at priming. In an independent study, 4 of 16 healthy donors (25%) responded to MART-1 (27–35) after two stimulations (27). Similarly, anti-p53 (264–272) cytolytic effectors were generated from 2 of 5 healthy donors (40%) after several restimulations with peptide-pulsed DCs (28), whereas anti-gp100 CTLs were elicited in 1 of 10 healthy donors at priming (29).

In contrast with melanoma Ag, the immunogenicity of which has been repeatedly investigated in extensive studies, the experience with E75 is limited. Similar to the MART-1 peptide, E75 activated rapid cytokine secretion from cultured ovarian tumor-infiltrating lymphocytes or CTL lines (30, 31) and activated cytolysis in tumor-associated lymphocytes (32). Freshly isolated PBMCs from cancer patients that were not vaccinated with peptide rapidly responded to E75 and to another HER-2-epitope, GP2 (33), in a similar fashion as melanoma patients to MART-1, by induction of specific cytolysis and tumor recognition (34). The ease by which E75- and GP2-specific cytolytic activities were induced in patients suggested that E75 and GP2 reactivated effector/memory CTLs rather than primed naïve cells (34). Our results showing 2 of 10 responders at priming (20%) and 5 of 10 responders at restimulation (50%) indicate that E75 is similar to MART-1 (27–35), tyrosinase (369–377), and p53 (264–272) in its ability to activate cytolysis in randomly selected healthy donors.

It is possible that E75 cannot elicit a complete response in all donors during PBMC priming. Our recent studies on cytokine responses by E75-primed PBMCs in healthy donors show that E75 rapidly activated specific IFN-γ release in five of six healthy donors, an effect that was enhanced by IL-12.[6] Although the same donors were used in these studies and IL-12 was used in parallel experiments, we could not observe a similar effect with respect to induction of cytolysis. Thus, a complete response (cytokines and cytolysis) was observed only in two donors of the six tested. This suggests that E75 may act as a partial agonist. In support of this possibility, Zaks and Rosenberg (1) reported recently that E75 vaccination in IFA of four cancer patients led to a peptide-specific response at restimulation in all patients (cytolysis and IFN-γ). T cells from two of three E75-vaccinated patients recognized, occasionally, tumor cells by specific IFN-γ secretion but failed to show specific tumor lysis (1). Similarly, tyrosinase 369–377-specific CTLs from two of four responders failed to recognize tyrosinase-expressing tumors (21). Preliminary results from a vaccine trial in breast and ovarian cancer patients indicate that PBMCs from only two of six E75-vaccinated patients (33%) stimulated *in vitro* elicited specific CTL activity against peptide and specific tumor recognition, although all responded to E75 by specific IFN-γ induction (2).

The similar response rates for induction of cytolysis in healthy donors to *in vitro* tumor peptide vaccination raise a number of questions about the application of this approach:

(*a*) Why, regardless of the Ag used, only 10–20% of healthy donors respond at priming, and only 40–50% at restimulation with

[5] T. V. Lee and C. G. Ioannides, preliminary data.

[6] T. V. Lee, B. W. Anderson, *et al.*, submitted for publication.

peptide? One possibility is that healthy donors have different precursor frequencies for the CTL epitopes, and repeated peptide stimulation (three to four times) is not sufficient to expand the effector population to sufficiently high numbers to detect CTL activity. Thus, one approach is to continue repeated vaccinations and deliver exogenous help by helper peptides and cytokines until such CTL responses are elicited (9, 35).

(*b*) If the 10–20% of donors that respond to peptide priming have higher pCTL frequencies for E75/MART-1 than nonresponders, then what is the reason for this increased frequency? It is tempting to speculate that local inflammatory conditions and cross-reactive priming may activate CTL precursors, such as in donor 9, and these precursors become tolerized.

(*c*) If pCTL frequency is similarly low in all healthy donors, then why do some respond better than others? One possibility is that discrete changes in HLA-A2 attributable to HLA-A2 polymorphism may lead to a more immunogenic E75 in some donors. In support of this possibility, Maurer *et al.* (36) demonstrated that mutated HLA-A2 in position 97 can segregate MART-1 (27–35)-induced cytolysis from cytokine production. Furthermore, the pool of E75 precursors may expand or contract over time because of different environmental factors (37, 38). This may be supported by the fact that the responders showed E75-specific CTL activity frequently, whereas some of the nonresponders showed activity only occasionally in the majority of independently performed experiments. Additional studies using carboxyfluorescein acetate to determine cell division, E75-tetramers to determine the frequency of E75-specific CTLs, and intracellular IFN-γ staining are required to distinguish among these possibilities.

Increased HLA-A2 binding affinity by COOH-terminal modification was able to enhance tumor Ag immunogenicity (in some instances), as shown in our previous studies (15) and by other investigators using the melanoma Ag gp100 (39, 40). However, increased HLA-A2 binding affinity does not always predict a higher T-cell receptor signaling or a complete T-cell activation (reviewed in Ref. 41). In some of the reported cases, CTLs induced by higher HLA-A2 affinity binding variant showed low affinity for the tumor cells (15), and more recently (39, 40), some reports have suggested that they preferentially targeted tumors expressing high numbers of the epitope. Thus, novel immunogens need to be designed with emphasis on modifications in the Ag that would induce high rates of proliferation and select responders of high cytolytic activity, *i.e.*, high catalytic activity as demonstrated by enzymes. Because restimulations may induce apoptosis, it remains to be seen whether tumor-specific CTL expansion would require several agonists, each being capable of activating one effector function at a time. At the present, the results of this study demonstrated that cytolytic effectors to an epitope on HER-2, which is overexpressed on the majority of epithelial tumors, can be elicited in a fraction of healthy individuals at priming, and in nonresponders, the precursors were not tolerized. Because these studies were performed with 10 donors and substantiated in multiple replicate induction experiments using the same stimulation system, this suggests that this stimulation system may be identifying individuals that will respond to vaccine with a tumor Ag. This may have implications for preventative vaccination in high-risk individuals.

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REFERENCES

1. Zaks, T. Z., Rosenberg, S. A. Immunization with a peptide epitope (p369–377) from HER-2/neu leads to peptide-specific cytotoxic T lymphocytes that fail to recognize HER-2/neu$^+$ tumors. Cancer Res., *58:* 4902–4908, 1998.

2. Anderson, B., Lu, J. J., Ibrahim, N., Hortobagyi, G., Brewer, H., Przepiorka, D., Ioannides, C., Grabstein, K., Cheever, M., and Murray, J. Immunomodulating effects of a HER2/neu derived HLA-A2 specific peptide E75 combined with GM-CSF in patients with metastatic breast and ovarian cancer. Proc. Am. Soc. Clin. Oncol., *18:* A1691, 1999.

3. Fisk, B., Blevins, T. L., Wharton, J. T., and Ioannides, C. G. Identification of an immunodominant peptide of *HER-2/neu* protooncogene recognized by ovarian tumor-specific cytotoxic T lymphocyte lines. J. Exp. Med., *181:* 2109–2117, 1995.

4. Hudson, J. M., Castilleja, A., Murray, J. L., Honda, T., Kudelka, A., Singletary, E., Wharton, J. T., and Ioannides, C. G. Growth and antigen recognition of tumor-infiltrating lymphocytes from human breast cancer. J. Interferon Cytokine Res., *18:* 529–536, 1998.

5. Dudley, M. E., Nishimura, M., I., Holt, A. K. C., and Rosenberg, S. A. Antitumor immunization with a minimal peptide epitope (G9–209-2M) leads to a functionally heterogeneous CTL response. J. Immunother., *22:* 288–298, 1999.

6. Carbone, E. R., Moore, M. W., Sheil, J. M., and Bevan, M. J. Induction of cytotoxic T lymphocytes by primary *in vivo* stimulation with peptides. J. Exp. Med., *167:* 1767–1779, 1988.

7. Kelly, J. M., Sterry, S. J., Cose, S., Turner, S. J., Fecondo, J., Rodda, S., Fink, P. J., and Carbone, F. R. Identification of conserved T cell receptor CDR3 residues contacting known exposed peptide side chains from a major histocompatibility complex class I-bound determinant. Eur. J. Immunol., *23:* 3318–3326, 1993.

8. Pittet, M., Valmori, D., Dunbar, P. R., Speiser, D. E., Lienard, D., Lejeune, F., Fleischhauer, K., Cerundolo, V., Cerottini, J-C., and Romero, P. High frequencies of naïve melan-A/MART-1-specific CD8$^+$ T cells in a large proportion of human histocompatibility leukocyte antigen (HLA)-A2-individuals. J. Exp. Med., *190:* 705–715, 1999.

9. Anichini, A., Molla, A., Mortarini, R., Tragni, G., Bersani, I., DiNicola, M., Gianni, A. M., Pilotti, S., Dunbar, R., Cerundolo, V., and Parmiani, G. An expanded peripheral T cell population to a cytotoxic T lymphocyte (CTL)-defined, melanocyte-specific antigen in metastatic melanoma patients impacts on generation of peptide-specific CTLs but does not overcome tumor escape from immune surveillance in metastatic lesions. J. Exp. Med., *190:* 651–667, 1999.

10. Marincola, F. M., Rivoltini, L., Salgaller, M. L., Player, M., and Rosenburg, S. A. Differential anti-MART-1/MelanA CTL activity in peripheral blood of HLA-A2 melanoma patients in comparison to healthy donors: evidence of *in vivo* priming by tumor cells. J. Immunother., *19:* 266–277, 1996.

11. Alexander-Miller, M. A., Leggatt, G. R., and Berzofsky, J. A. Selective expansion of high- or low-avidity cytotoxic T lymphocytes and efficacy for adoptive immunotherapy. Proc. Natl. Acad. Sci., *93:* 4102–4107, 1996.

12. Ioannides, C. G., Fisk, B., Jerome, K. R., Chesak, B., Irimura, T., Wharton, J. T., and Finn, O. J. Ovarian tumors reacting cytotoxic T lymphocytes can recognize peptide determinants on polymorphic epithelial mucins. *In:* F. Sharp, P. Mason, J. Berek, and A. Blackett (eds.), Ovarian Cancer, pp. 317–330. London: Chapman and Hall, 1994.

13. Sallusto, F., and Lanzavecchia, A. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor. J. Med. Exp., *179:* 1109–1118, 1994.

14. Pickl, W. F., Majdic, O., Kohl, P., Stockl, J., Riedl, E., Scheinecker, C., Bello-Fernandez, C., and Knapp, W. Molecular and functional

characteristics of dendritic cells generated from highly purified CD14$^+$ peripheral blood monocytes. J. Immunol., *157:* 3850–3859, 1996.

15. Fisk, B., Chessak, B., Pollack, M., Wharton, J. T., and Ioannides, C. G. Oligopeptide induction of a cytotoxic T lymphocyte response to *HER-2/neu* proto-oncogene *in vitro*. Cell. Immunol., *157:* 412–427, 1994.

16. Ioannides, C. G., Platsoucas, C. D., Rashed, S., Wharton, J. T., Edwards, C. L., and Freedman, R. S. Tumor cytolysis by lymphocytes infiltrating ovarian malignant ascites. Cancer Res., *51:* 4257–4265, 1991.

17. Gorelik, L., Bar-Dagan, Y., and Mokyr, M. B. Insight into the mechanism(s) through which TNF promotes the generation of T cell-mediated antitumor cytotoxicity by tumor bearer splenic cells. J. Immunol., *156:* 4298–4308, 1996.

18. Mehrotra, P. T., Wu, D., Crim, J. A., Mostowski, H. S., and Siegel, J. P. Effects of IL-12 on the generation of cytotoxic activity in human CD8$^+$ T lymphocytes. J. Immunol., *151:* 2444–2452, 1993.

19. Bhardwaj, N., Seder, R. A., Reddy, A., and Feldman, M. V. IL-12 in conjunction with dendritic cells enhances antiviral CD8+ CTL responses *in vitro*. J. Clin. Investig., *98:* 715–722, 1996.

20. Guerder, S., Carding, S. R., and Flavell, R. A. B7 costimulation is necessary for the activation of the lytic function in cytotoxic T lymphocyte precursors. J. Immunol., *155:* 5167–5174, 1995.

21. Visseren, M. J. W., van Elsas, A., van der Voort, E. I. H., Ressing, M. E., Kast, W. M., Schrier, P. I., and Melief, J. M. ACTL specific for the tyrosinase autoantigen can be induced from healthy donor blood to lyse melanoma cells. J. Immunol., *154:* 3991–3998, 1995.

22. Bakker, A. B. H., Marland, G., de Boer, A. J., Huijbens, R. J. F., Danen, E. H. J., Adema, G. J., and Figdor, C. G. Generation of antimelanoma cytotoxic T lymphocytes from healthy donors after presentation of melanoma-associated antigen-derived epitopes by dendritic cells *in vivo*. Cancer Res., *55:* 5330–5334, 1995.

23. Perez-Diez, A., Butterfield, L. H., Li, L., Chakraborty, N. G., Economou, J. S., and Mukherji, B. Generation of CD8$^+$ and CD4$^+$ T-cell response to dendritic cells genetically engineered to express the *MART-1/Melan-A* gene. Cancer Res., *58:* 5305–5309, 1998.

24. Butterfield, L. H., Jilani, S. M., Chakraborty, N. G., Bui, L. A., Ribas, A., Dissette, V. B., Lau, R., Gamradt, S. C., Glaspy, J. A., McBride, W. H., Mukherji, B., and Economou, J. S. Generation of melanoma-specific cytotoxic T lymphocytes by dendritic cells transduced with a MART-1 adenovirus. J. Immunol., *161:* 5607–5613, 1998.

25. Zajac, P., Oertli, D., Spagnoli, G. C., Noppen, C., Schaefer, C., Heberer, M., and Marti, W. R. Generation of tumoricidal cytotoxic T lymphocytes from healthy donors after in stimulation with a replication into competent vaccinia virus encoding MART-1/Melan-A 27–35 epitope. Int. J. Cancer, *71:* 491–496, 1997.

26. Zajac, P., Schutz, A., Oertli, D., Noppen, C., Schaefer, C., Heberer, M., Spagnoli, G. C., and Marti, W. R. Enhanced generation of cytotoxic T lymphocytes using recombinant vaccinia virus expressing human tumor-associated antigens and B7 costimulatory molecules. Cancer Res., *58:* 4567–4571, 1998.

27. Chen, Q., Jackson, H., Gibbs, P., Davis, I. D., Trapani, J., and Cebon, J. Spontaneous T cell responses to melanoma differentiation antigens from melanoma patients and healthy subjects. Cancer Immunol. Immunother., *47:* 191–197, 1998.

28. Chikamatsu, K., Nakano, K., Storkus, W. J., Appella, E., Lotze, M. T., Whiteside, T. L., and DeLeo, A. B. Generation of anti-p53 cytotoxic T lymphocytes from human peripheral blood using autologous dendritic cells. Clin. Cancer Res., *5:* 1281–1288, 1999.

29. Jager, E., Ringhoffer, M., Arand, M., Karbach, J., Jager, D., Ilsemann, C., Hagedorn, M., Oesch, F., and Knuth, A. Cytolytic T cell reactivity against melanoma-associated differentiation antigens in peripheral blood of melanoma patients and healthy individuals. Melanoma Res., *6:* 419–425, 1996.

30. Wasserman, K., Corsi, M. M., Szekely, L., Kono, K., Maes, H. H., and Kiessling, R. Conversion of *in vitro* cultured human monocytes into effective presenters of an HER2/neu-encoded CTL peptide epitope. Scand. J. Immunol., *45:* 678–682, 1996.

31. Ferlazzo, G., Wesa, A., Wei, W. Z., and Galy, A. Dendritic cells generated either from CD34+ progenitor cells or from monocytes differ in their ability to activate antigen-specific CD8+ T cells. J. Immunol., *163:* 3597–3604, 1999.

32. Rongcun, Y., Salazar-Onfray, F., Charo, J., Malmberg, K. J., Evrin, K., Maes, H., Kono, K., Hising, C., Petersson, M., Larsson, O., Lan, L., Appella, E., Sette, A., Celis, E., and Kiessling, R. Identification of new HER2/neu-derived peptide epitopes that can elicit specific CTL against autologous and allogeneic carcinomas and melanomas. J. Immunol., *163:* 1037–1044, 1999.

33. Peoples, G. E., Goedegebuure, P. S., Smith, R., Linehan, D. C., Yoshino, I., and Eberlein, T. J. Breast and ovarian cancer-specific cytotoxic T lymphocytes recognize the same HER2/neu-derived peptide. Proc. Natl. Acad. Sci. USA, *92:* 432–436, 1995.

34. Brossart, P., Stuhler, G., Flad, T., Stevanovic, S., Rammensee, H. G., Kanz, L., and Brugger, W. HER-2/neu-derived peptides are tumor-associated antigens expressed by human renal cell and colon carcinoma lines and are recognized by *in vitro* induced specific cytotoxic T lymphocytes. Cancer Res., *58:* 732–736, 1998.

35. Ostankovitch, M., Le Gal, F. A., Connan, F., Chassin, D., Choppin, J., and Guillet, J. G. Generation of Melan-A/MART-1-specific CD8+ cytotoxic T lymphocytes from human naïve precursors: helper effect requirement for efficient primary cytotoxic T lymphocyte induction *in vivo*. Intl. J. Cancer, *72:* 987–994, 1997.

36. Maeurer, M. J., Chan, H. W., Karback, J., Salter, R. D., Knuth, A., Lotze, M. T., and Storkus, W. J. Amino acid substitutions at position 97 in HLA-A2 segregate cytolysis from cytokine release in MART-1/Melan-A peptide AAGIGILTV-specific cytotoxic T lymphocytes. Eur. J. Immunol., *26:* 2613–2623, 1996.

37. Goldrath, A. W., and Bevan, M. J. Selecting and maintaining a diverse T-cell repertoire. Nature (Lond.), *402:* 255–262, 1999.

38. Lalvani, A. R., Brookes, S., Hambelton, J. J., Britton, A. V. S., Hill, A., and McMichael, A. J. Rapid effector function in CD8+ memory T cells. J. Exp. Med., *186:* 859–865, 1997.

39. Clay, T. M., Custer, M. C., McKee, M. D., Parkhurst, M., Robbins, P. R., Kerstann, K., Wunderlich, J., Rosenberg, S. A., and Nishimura, M. I. Changes in the fine specificity of gp100(209–217)-reactive T cells in patients following vaccination with a peptide modified at an HLA-A2.1 anchor residue. J. Immunol., *162:* 1749–1755, 1999.

40. Lee, K-H., Panelli, M. C., Kim, C. J., Riker, A. I., Betinoti, M. P., Roden, M. M., Fetsch, P., Abati, A., Rosenberg, S. A., and Marincola, F. M. Functional dissociation between local and systemic immune response during anti-melanoma peptide vaccination. J. Immunol., *161:* 4183–4194, 1998.

41. Germain, R. N., and Stefanova, I. The dynamics of T cell receptor signaling: complex orchestration and the key roles of tempo and cooperation. Annu. Rev. Immunol, *17:* 467–522, 1999.

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Secretion of CXC Chemokine IP-10 by Peripheral Blood Mononuclear Cells from Healthy Donors and Breast Cancer Patients Stimulated with HER-2 Peptides

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ABSTRACT

CXC chemokines play an important role in recruitment of T cells to the site of activation and regulation of angiogenesis. CXC chemokines are secreted by T cells stimulated with cytokines or by established cytotoxic T lymphocyte (CTL) lines at recognition of conventional antigen (Ag), but the activation requirements and the relationship of interferon-γ (IFN-γ) inducible protein (IP-10) secretion with IFN-γ induction in lymphocytes are still unclear. We studied the induction of IP-10 from nonadherent peripheral blood mononuclear cells (PBMC) by IFN-γ, interleukin-12 (IL-12), and the HER-2 peptide E75, which forms a CTL-defined antigen. We found that IFN-γ alone was a weak inducer of IP-10 in these cells, whereas IL-12 was a significantly stronger inducer of IP-10. In the presence of IL-12, the tumor peptide E75 (HER-2, 369–377) was a stronger inducer of IP-10 than was IL-12 alone. E75 and its variants mutated at position 5 could also induce IP-10 in the absence of exogenous IL-12 or IFN-γ. IP-10 induction by E75 required HLA-A2 presentation and B7-CD28 interactions and was partially inhibited by blocking of CD40-CD40L interactions. These results indicate that presentation of tumor peptides to peripheral T cells can induce a fast chemokine response, which in its early phase may be higher than the IFN-γ response. This shows that the IP-10 response was independent of any early-phase IFN-γ response in peripheral T cells. This may be important for understanding the regulation of the balance between chemoattractant chemokines (CC) and CXC chemokines by tumor Ag and may have implications for understanding the mechanisms of polarization of T cells and conditioning of antigen-presenting cells (APC) by tumor antigens.

INTRODUCTION

DATA FROM BOTH HUMAN STUDIES and animal models suggest that cytotoxic T lymphocytes (CTL) are an important host defense mechanism against tumors. Recent studies, as well as a number of clinical trials, have demonstrated that CTL are capable of expansion *in vivo* and are associated with the control of tumor growth. The fact that tumor cell growth can be controlled by $CD8^+$ CTL is indicative of the potency of these cells. It has been established that after activation, T cells acquire effector functions. For $CD8^+$ T cells, two functions have been extensively characterized: the specific lysis of antigen (Ag)-expressing targets and cytokine secretion in response to the specific antigen.

A recently identified novel $CD8^+$ CTL function is the release of CXC chemokines in response to cognate Ag.[1,2] This function was defined by the ability of established $CD8^+$ CTL lines to attract, by secretion of interferon-γ (IFN-γ) inducible protein (IP-10), other cells, particularly Ag-specific $CD4^+$ cells.[3] Regulation of this chemoattractive function appears to be an intricate process involving specific chemokines and chemokine receptors.[2] Expression of the latter provides a signal code for selective migration of subsets of effector and regulatory cells at the site of inflammatory reaction and up-regulation and downregulation of the immune response to a specific antigen.[2,4] In addition to their role as chemoattractants for leukocytes, CXC chemokines play a role in regulating angiogenesis and initiate signaling events in T lymphocytes, although

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with a few exceptions; for example, interleukin-8 (IL-8) is still poorly understood.[2] CXC chemokines are coordinately secreted in response to cytokines, thus exposing the tumor environment to factors that may promote or inhibit tumor growth.[5] Members of the CXC chemokine family can behave as either angiogenic or angiostatic factors.[6] Such chemokines as IP-10 monokine induced by IFN-γ (MIG), and platelet factor-4 (PF-4), which lack a three amino acid motif (Glu-Leu-Arg, i.e., ELR) in the primary sequence, are angiostatic factors. They are also defined as CXC-non-ELR chemokines. In contrast, CXC chemokines that contain this motif, such as IL-8, are angiogenesis-inducing factors.[6]

CXC-non-ELR chemokines, such as IP-10 and MIG, can inhibit angiogenesis and mediate tumor regression.[5] There is little knowledge about how the production of these chemokines from lymphocytes can be enhanced and sustained. Understanding this question is important because IP-10 mediates its endothelial cell growth inhibition and signaling effects at relatively high concentrations (nanomolar range).[7] Original studies showed that IP-10 (and MIG) can be secreted by monocytes, fibroblasts, and endothelial cells after stimulation with tumor necrosis factor-α (TNF-α) $\pm$ IFN-γ. Tumor cells were also shown to produce the angiostatic chemokine IP-10 constitutively[6] or after treatment with IL-12,[8,9] but the IP-10 levels were significantly lower than those produced by CTL lines.[3] Production of IP-10 from a number of nonlymphoid cells required high concentrations of IFN-γ as an inducer. The amounts of IFN-γ needed to induce IP-10 gene expression are in the range between 1 and 15 ng/ml.[10,11] Thus, one approach to enhanced IP-10 production is to increase the amount of IFN-γ used as inducer or to add synergistic factors along with IFN-γ, such as TNF-α, lipopolysaccharide (LPS), or phorbol myristate acetate (PMA).[10,12,13] The synergistic antitumor effects of IL-12 and IL-18 reflect, among others, inhibition of angiogenesis.[14] These effects appear to be mediated at least in part by IP-10.[15] However, IL-12 and IL-18 also act either as inducers of IFN-γ or costimulators of its production.[16] Attempts to enhance immune cell IFN-γ production by increasing the concentration of its inducer (IL-12) led to immune suppression as a result of IFN-γ induction of nitric oxide synthase (iNOS) as well as to high IL-12 dose-related toxicities, raising concerns about the efficiency of this approach.[17] Thus, alternative approaches to increased concentrations of induced cytokines are needed to stimulate angiostatic chemokine production.

Although a direct, nonimmune function of IP-10 in the inhibition of tumor growth has been described,[9] several studies reported a T cell dependency of the antitumor activity of IP-10.[18] Recently, IP-10 was shown to be secreted by activated CD8$^+$ CTL lines after stimulation with Ag.[3,19] However, there are no studies that address the activation requirements for IP-10 production by T cells. It is still unclear whether CD8$^+$ cells secrete IP-10 at priming with Ag or whether IP-10 production is a late effect following cell expansion and differentiation in response to Ag. It is also unknown whether in addition to conventional Ag, tumor Ag can activate IP-10 production in CD8$^+$ cells directly or whether IP-10 is a secondary effect to Th1 cytokine induction (e.g., IFN-γ) and the presence of activated Th1 cells, which were reported to secrete CXC chemokines.[20]

We found that exogenous IFN-γ induced IP-10 in lymphocytes with slow kinetics. In contrast, the kinetics of induction of IL-10 by IL-12 was significantly faster. Stimulation of peripheral blood mononuclear cells (PBMC) or of isolated CD8$^+$ cells from healthy donors and breast cancer patients with the HER-2 peptide E75 (369–377)[21] rapidly induced IP-10 within 24 h, even in the absence of exogenous IL-12 or IFN-γ or both. Freshly isolated PBMC from both healthy donors and breast cancer patients rapidly secreted high levels of IP-10 in response to tumor Ag (peptide) without previous restimulation and culture. The ability of tumor peptide E75 to induce IP-10 from T cells was modulated by changes in its sequence, suggesting T cell receptor (TCR) signaling may define a pathway for IP-10 induction.

MATERIALS AND METHODS

Synthetic peptides

The following synthetic peptides were used in these experiments: E75, KIFGSAFL; F42, KIFGKLAFL; F43, KIFGALAFL; and F45, KIFGGLAFL. E75 maps an immunodominant CTL epitope in HER-2 and is frequently recognized by ovarian and breast tumor-associated CTL.[21–23] The HER-2 peptide G89 (HER-2, 777–789) was used as a helper peptide.[24] All peptides were prepared by the Synthetic Antigen Laboratory of M.D. Anderson Cancer Center by a solid-phase method and purified by HPLC.

Cells

PBMC were obtained from healthy donors and breast cancer patients. Expression of HLA-A2 was verified by immunofluorescence with anti-HLA-A2, monoclonal antibody (mAb) BB7.2. Dendritic cells (DS) were generated from plastic adherent PBMC by standard culture in granulocyte-monophage colony-stimulating (GM-CSF) and IL-4 for 5 days as described.[25] The DC were detached by gentle pipetting, washed, and pulsed with various concentrations of peptides. CD8$^+$ cells were separated by negative selection from PBMC using mAb to CD8, CD16, CD56, and magnetic beads (Dynabead) coated with anti-mouse mAb. PBMC or isolated CD8$^+$ cells were stimulated with DC pulsed with E75 or its variants over a range of concentrations at a stimulator:responder ratio of 1:25.

Cytokines

The following cytokines were used in these experiments: IFN-γ (sp. act. 4×10^7 U/mg) (PharMingen, San Diego, CA), IL-12 (sp. act. 5×10^6 U/mg) (PharMingen), IP-10 (sp. act. 5×10^5 U/mg) (R&D Systems, Minneapolis, MN), TNF-α (sp. act. 2.5×10^7 U/mg) (Cetus, Emeryville, CA) IL-2 (sp. act. 18×10^6 U/mg)(Cetus).

Antibodies

The following antibodies were used in these experiments for blocking: anti-IFN-γ, anti-CD40L, and anti-CD40 (PharMingen) anti-HLA-A2 (B7.2) (ATCC, Rockville, MD), FITC-conjugated anti-CD8 and anti-CD4 were obtained from Ortho (Raritan, NJ).

IP-10 detection

The ability of cells to secrete IP-10 in response to cytokines and peptides was determined by culturing nonadherent PBMC or isolated $CD8^+$ cells or both and collecting supernatants at corresponding times. The levels of IP-10 secreted were determined using a modified sandwich ELISA (R&D Systems) established in our laboratory. A flat-bottom 96-well microtiter plate was coated with 100 μl/well of antihuman IP-10 mAb (2 μg/ml in phosphate-buffered saline [PBS], pH 7.2) (R&D) for 24 h at ambient temperature. The plate was subsequently washed with PBS, pH 7.4, and 0.05% Tween-20, then blocked with 3% ovalbumin (Sigma Chemical Co., St. Louis, MO), 5% sucrose, and 0.05% NaN_3. IP-10 standards were made from recombinant human IP-10 (rHuIP-10) (R&D) in a solution consisting of Tris-buffered saline (TBS), pH 7.3, 0.05% Tween-20, and 0.1% bovine serum albumin (BSA) using serial dilutions. Standards or supernatants (100 μl/well) were plated in duplicate and incubated at ambient temperature for 2 h. After three washes, 100 μl/well of biotinylated antihuman IP-10 mAb (100 ng/ml in TBS, pH 7.3, 0.1% BSA) was added, followed by 100 μl/well of streptavidin-peroxidase conjugate. The chromogen substrate, at 100 μl/well, consisted of DMSO and H_2SO_4. Plates were read at 450 nm in an automated microplate reader (Bio-Tek Instruments, Inc., Richmond, CA). Standards dilutions of IP-10 ranged from 4000 pg/ml to 15.6 pg/ml. This method consistently detected IP-10 concentrations >31.25 pg/ml in a linear fashion. IFN-γ was also determined by ELISA, using a specific ELISA kit (R&D).

RESULTS

Kinetics of IP-10 induction by IFN-γ and IL-12 in plastic nonadherent PBMC

To address the question whether IFN-γ can induce IP-10 production directly in lymphocytes, plastic nonadherent PBMC (monocyte-depleted PBMC) from a healthy donor were incubated with IFN-γ over a range of concentrations, 0–100 U/ml, and the supernatants were collected and used for determination of IP-10 by ELISA. The specific activity of IFN-γ corresponded to 40 U/ng. The results in Figure 1A,B show a steady increase in IP-10 secretion over a 120-h incubation period. IP-10 was not detected in response to low concentrations of IFN-γ (5 U/ml) but was present with its levels increasing over time in response to 100 U/ml IFN-γ. This suggests that IFN-γ alone at concentrations of 50–100 U/ml, that is, 1.2–2.5 ng/ml, is a weak inducer of IP-10 in PBMC. To determine if IP-10 induction was mediated through IFN-γ, the experiment was repeated in the presence of IFN-γ-specific mAb. Anti-IFN-γ completely inhibited IP-10 production, suggesting that IP-10 is induced by IFN-γ (Fig. 1C).

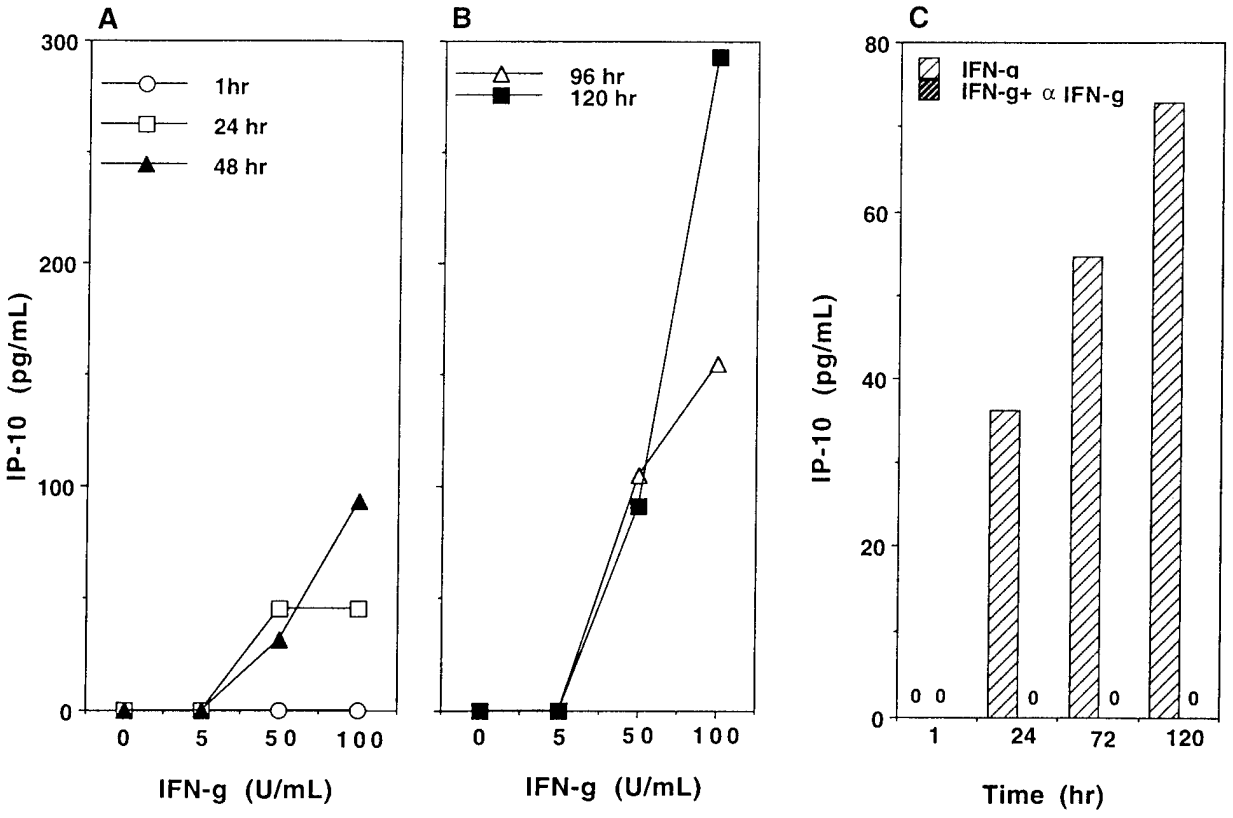

FIG. 1. (A,B) Time course and concentration-dependent kinetics of IP-10 induction by IFN-γ from plastic nonadherent PBMC isolated from a healthy donor. (C) Inhibition of IP-10 induction by mAb to IFN-γ. IFN-γ was used at 50 U/ml. Anti-IFN-γ was used at 2.5 μg/ml. IP-10/pg/ml represents the amount of IP-10 produced by 10^6 cells.

Because IFN-γ induction is dependent in many instances on IL-12 as a cofactor,[16] we investigated whether IL-12 by itself can induce IP-10 and IFN-γ (Fig. 2A,B). Plastic nonadherent PBMC from another healthy donor (donor 2, HLA-A2$^+$) were used as responders. At 24 h, IL-12 at both 100 and 1000 pg/ml, that is, and 10 U/ml, had a weak IP-10-inducing and IFN-γ-inducing effect. At 72 h after IL-12 stimulation, the induced IFN-γ concentration was in the range of 50 pg/ml. This is below the levels required (1.2–2.5 ng) (Fig. 1) to induce IP-10 from PBMC. In contrast, IL-12 had a dramatic effect on IP-10 induction from these cells at 72 h. This effect was IL-12 concentration dependent (Fig. 2B). To address the question of the ability of similar concentrations of IFN-γ and IL-12 to induce IP-10 from ovarian tumors, the ovarian cell line SKOV3.A2 was treated with 50 U/ml IFN-γ and 10 U/ml IL-12. The results in Figure 2C show that IFN-γ + IL-12 induced low levels of IP-10 in SKOV3.A2. The IP-10 levels increased slightly over a 5-day period but continued to be lower than the levels of IP-10 induced by IL-12 in lymphocytes (Fig. 2C). It should also be mentioned that SKOV3.A2 cells proliferated and increased in number over the 5-day culture period. Thus, a part of the increased levels of IP-10 may be attributed to increased numbers of IP-10-secreting cells.

CTL epitope E75 (HER-2, 369–377) rapidly induces IP-10 in the presence of IL-12

To determine if stimulation with tumor Ag, such as HER-2, induces IP-10, we used as responders plastic nonadherent PBMC from another HLA-A2$^+$ healthy donor (donor 3). These PBMC were stimulated with E75 pulsed on autologous DC in the presence or absence of IL-12. The results are shown in Figure 3. E75 in the presence of IL-12 rapidly induced IFN-γ within 24 h. The IFN-γ levels increased during the following 2 days. Again, IL-12 alone showed a weak ability to induce IFN-γ (Fig. 3A,C). At 100 pg/ml IL-12, IFN-γ was essentially undetectable, and the IFN-γ levels induced in the presence of 1000 pg/ml IL-12 were only slightly higher. IFN-γ was detected at 72 h and only in the presence of high concentrations of IL-12. In contrast, the pattern of IP-10 induction by IL-12 alone was similar to the pattern shown in Figure 2. IP-10 was induced by E75 in the presence of exogenous IL-12 with significantly faster kinetics than by IL-12 alone. Induction of IP-10 was also dependent on the concentration of exogenous IL-12. The level of IP-10 induced by E75 and IL-12 increased rapidly at 24 h. This increase was similar in the presence of either 1 or 10 U/ml IL-12 and was followed by a decline on day 3. The kinetics of

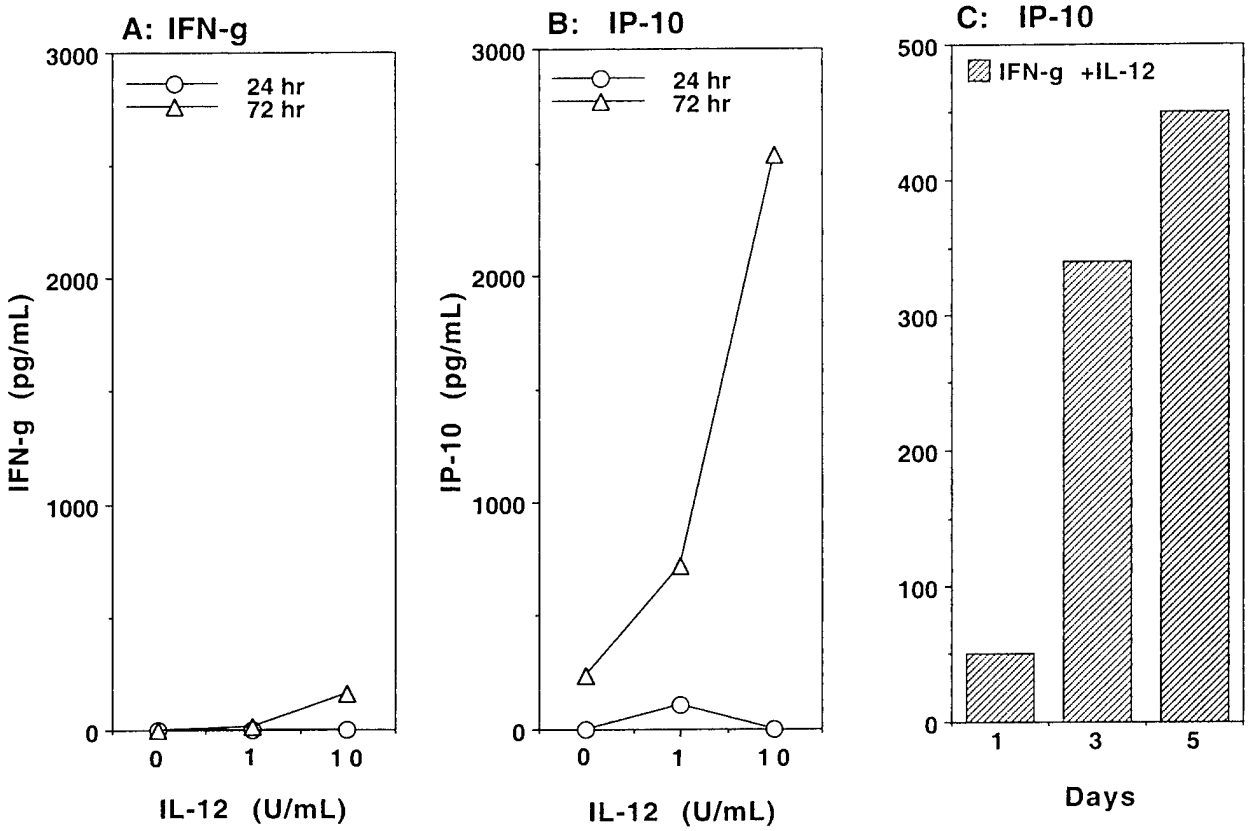

FIG. 2. (A,B) Time course and concentration-dependent induction of IFN-γ and IP-10 by IL-12. (C) Induction of IP-10 by IFN-γ and IL-12 from the ovarian cell line SKOV3.A2; 10^5 SKOV3.A2 cells were plated and used as responders. IFN-γ and IL-12 were added at initiation of the cultures (day 0) and on days 2 and 4. Supernatants were collected on days 0, 1, 3, and 5. SKOV3.A2 cells proliferated in these cultures. Thus, their final numbers exceeded 10^5.

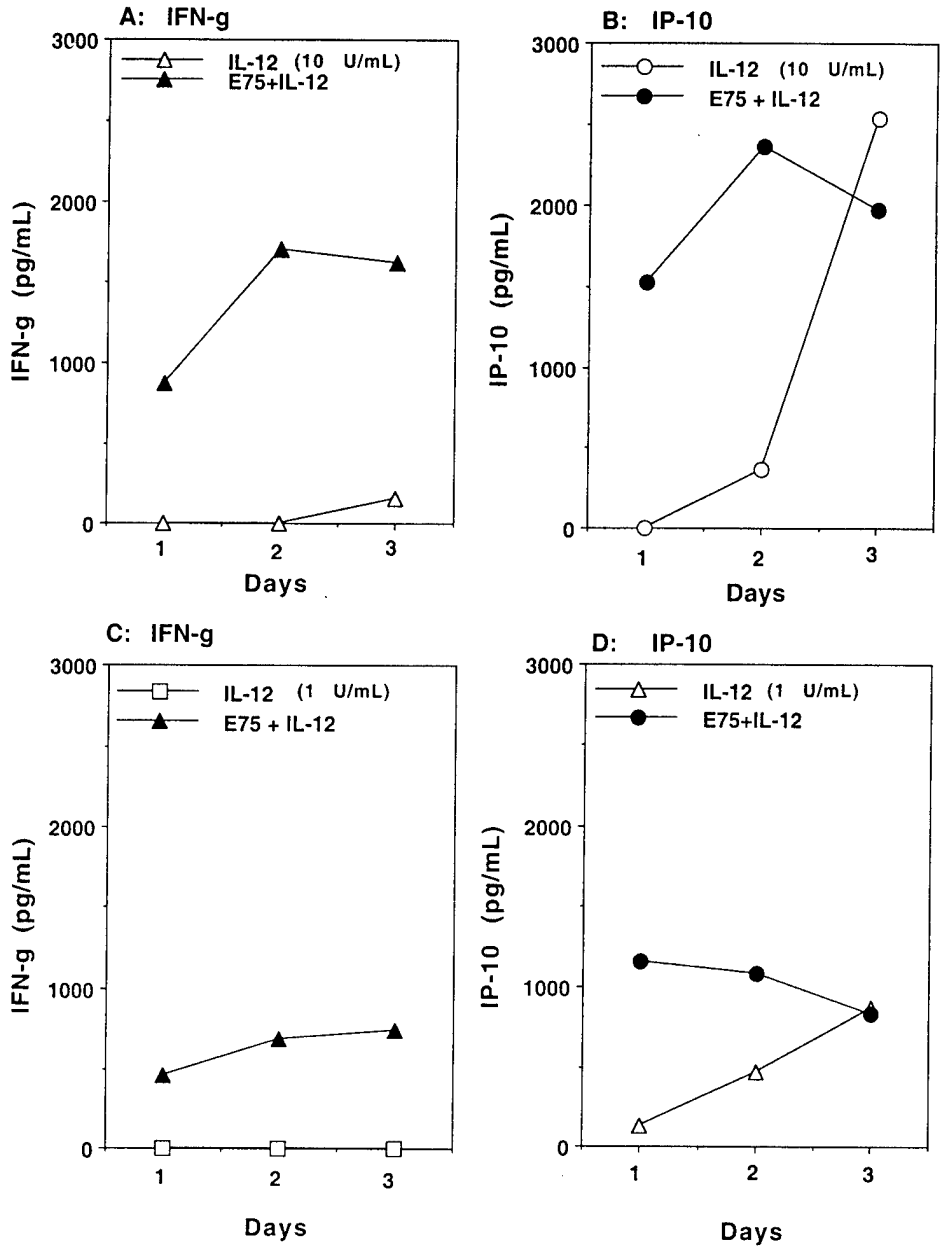

FIG. 3. Induction of IFN-γ and IP-10 by stimulation of HLA-A2$^+$ PBMC with autologous monocyte-derived DC, pulsed with E75 + IL-12. Results indicate IFN-γ and IP-10 levels/10^6 responders. IL-12 was used at 10 U/ml (**A,B**) and 2 U/ml (**C,D**). E75 was used at 100 μg/ml.

induction by E75 and IL-12 was fast. Afterward, the IP-10 levels slightly declined. IL-12 alone induced IP-10 more slowly than E75 + IL-12, but after 3 days, the levels of IP-10 were similar in both stimulation systems. In fact, the entire amount of IP-10 detected on day 3 could be accounted for by its induction by IL-12 alone. Thus, the tumor peptide and IL-12 induced IP-10 with different kinetics than did IL-12 alone. The levels of induced IP-10 did not correlate with the levels of endogenous IFN-γ induced by E75 + IL-12, suggesting that in T cells, IP-10 may be induced by E75 + IL-12 by different mechanisms than by IFN-γ.

To address the question whether IP-10 is induced by E75 in CD8$^+$ cells, we isolated CD8$^+$ cells from donor 2 and determined their ability to secrete IP-10 in response to E75 in the presence or absence of IL-12. The results are shown in Figure 4A,B. In the presence of IL-12, E75 induced IP-10 in a concentration-dependent fashion (Fig. 4A). Furthermore, E75 induced IP-10 from CD8$^+$ cells of donor 2 within 24 h even in the absence of exogenous IL-12 (Fig. 4B), but the IP-10 levels were significantly lower than in the presence of IL-12. Similar results were obtained with PBMC from an HLA-A2$^+$ breast cancer patient stimulated with E75 in the absence of IL-12 (Fig. 4C). Similarly, in this patient, induction of IP-10 by E75 did not require exogenous IL-12. Of interest, IP-10 production was

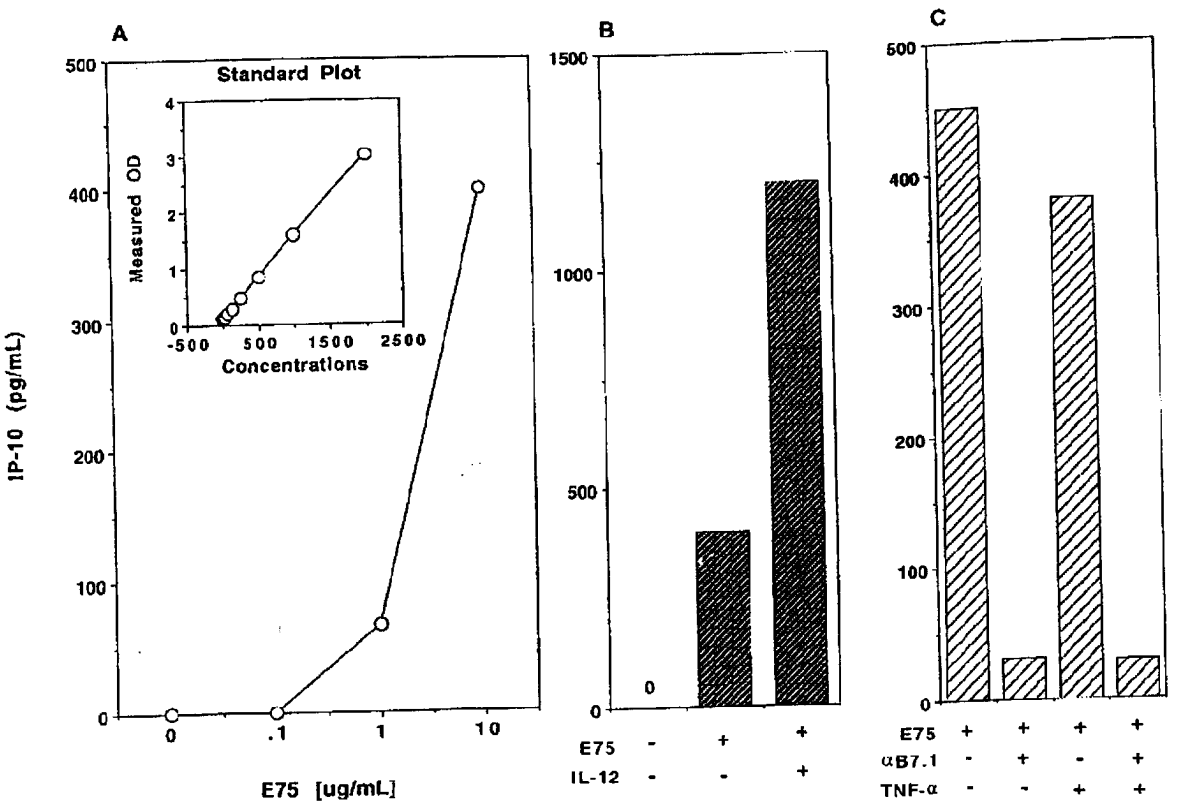

FIG. 4. (A) E75 concentration-dependent induction of IP-10. Responders were isolated $CD8^-$ cells. IL-12 was used at 3 U/ml (300 pg/ml). (B) E75 can induce IP-10 in the absence of exogenous IL-12. Responders were isolated $CD8^+$ cells. (C) IP-10 induction by E75 in the absence of exogenous IL-12 requires B7-CD28 interactions. Responders were isolated $CD8^-$ cells from an HLA-$A2^+$ breast cancer patient. TFN-α was used in a parallel experiment at 50 U/ml. (A,B,C) E75 was used at 25 μg/mL. All supernatants were collected at 24 h after stimulation with autologous E75 pulsed DC. (A) (Inset) A standard plot of OD_{450} versus IP-10 concentration established in our laboratory.

inhibited by anti-B7.1 mAb, suggesting a requirement for both Ag recognition and costimulation provided by DC in IP-10 induction. In these experimental conditions, TNF-α at 2 ng/ml did not enhance IP-10 induction.

To determine the fine specificity of E75 recognition for IP-10 induction, the experiments were repeated with donor 3. We used as stimulators E75 and three E75 analogs, F42, F43, and F45. In the mutated analogs, serine (Ser) at position (P) 5 was replaced with lysine (K5) in F42, with alanine (A5) in F43 and glycine (G5) in F45. Alanine and glycine differ from serine by the lack of a hydroxyl (HO) and a hydroxymethylene (CH_2-OH) group, respectively. In contrast, in lysine in F42, the HO group is substituted by a positively charged amino group. The results show that both F43 and F45 induced higher levels of IP-10 than E75 or F42. Thus, in this donor, the HO side chain of serine is significant for controlling IP-10 induction, as its removal enhanced IP-10 secretion (Fig. 5A,B).

To address the effects of a helper HER-2 peptide, G89, on IP-10 induction by E75, donor 3 PMBC were stimulated with autologous DC pulsed with E75 or E75 + G89. The results are shown in Figure 5C. Presentation of G89 significantly increased the levels of IP-10 produced by donor 3 PBMC, suggesting that G89 can help in IP-10 induction. In $CD8^+$ cells from the same donor, IP-10 was also induced by E75 (Fig. 5D) but at slightly lower levels when compared with unseparated lymphocytes.

Induction of IFN-γ and IP-10 by E75 requires CD40L interactions and HLA-A2 recognition

To address the question of the involvement of the surface receptors on antigen-presenting cells (APC) in IFN-γ and IP-10 induction, we investigated the effects of blocking the CD40-CD40L interaction, which is the main pathway of IL-12 induction by T cells.[26,27] Plastic nonadherent PBMC from donor 2 were stimulated with E75 at high concentrations (100 μg/ml) pulsed on autologous DC and as control with autologous DC that were not pulsed with peptide. IL-12 was not added to the cultures. The high concentration of E75 was used to ensure that E75 induced IFN-γ in the absence of exogenous IL-12 (T.V. Lee and B.W. Anderson, unpublished observations). The cultures were treated either with anti-CD40L mAb or with an isotype control Ab. The results are shown in Figure 6A,B. The results show that anti-CD40L mAb added in the induction phase inhibited almost completely (99%) IFN-γ production during the

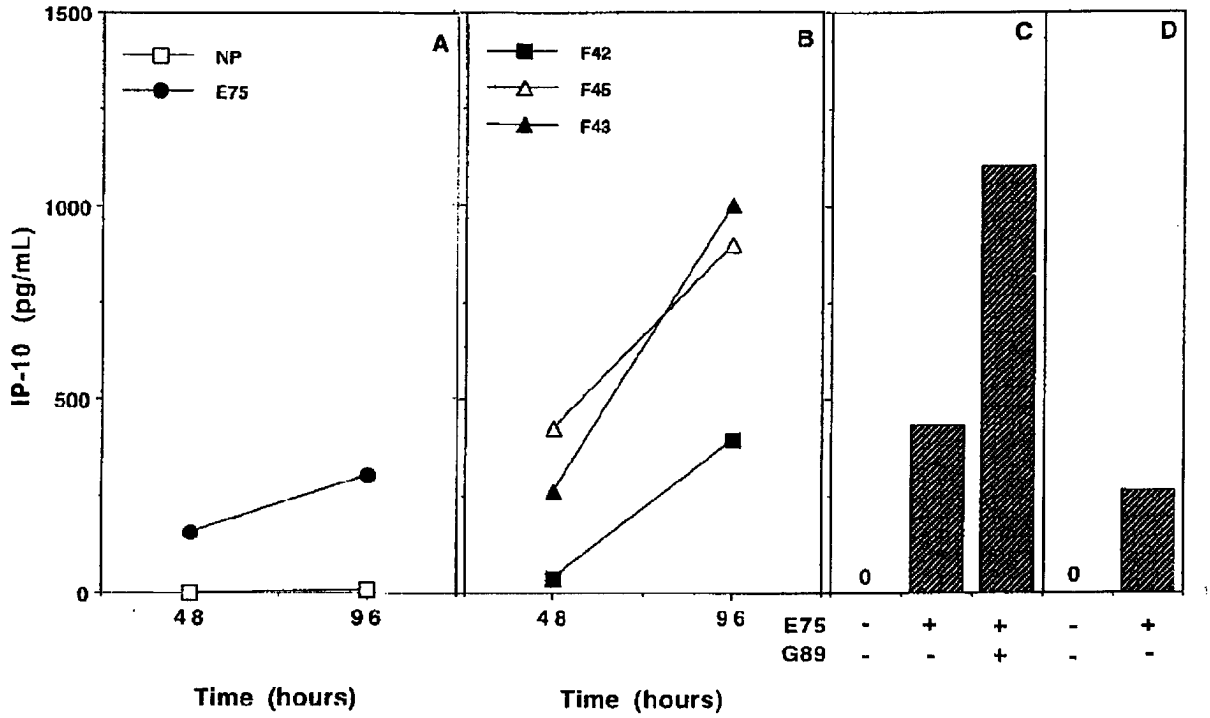

FIG. 5. (A,B) Antigen side chain-specific IP-10 induction from HLA-A2$^+$ PBMC. The sequences of E75 and its variants, F42, F43, and F45, are shown in Materials and Methods. All peptides were used at 25 μg/ml. NP, no peptide. (C,D) Helper HER-2 peptide G89 can increase levels of IP-10 induction, indicating the effect on CD4$^+$ cells of IP-10 secretion. In isolated CD8$^-$, E75 induced IP-10 secretion as previously noted, albeit at lower levels when compared with mixed lymphocyte induction. Autologous DC were used as stimulators at a ratio of 1:25 with responders. Results indicate IP-10 production/10^6 cells.

first 24 h. However, anti-CD40L did not inhibit IP-10 induction by E75 to a similar extent. IP-10 was inhibited by anti-CD40L by only 65%. The patterns of inhibition of IFN-γ and IP-10 production by anti-CD40L were divergent. The inhibitory effects of anti-CD40L on IFN-γ secretion diminished during the next 2 days but increased with regard to IP-10 production apparently because the IP-10 level in the absence of anti-CD40L is decreased. Because IFN-γ and IP-10 levels were determined from the same supernatants collected from the same wells of the same experiment, these results suggest that IP-10 induction by Ag in lymphocytes may not be entirely dependent on IFN-γ.

To determine if IP-10 induction requires recognition of tumor peptide in the context of HLA-A2, the experiment was repeated in the presence or absence of anti-HLA-A2 mAb, BB7.2, and of mAb to CD40L and CD40. The results in Figure 6C show that BB7.2 inhibited IP-10 production by 60%, indicating that IP 10 induction required recognition of an Ag presented by HLA-A2. Similarly anti-CD40L inhibited IP-10 induction by only 35%, confirming the results of the previous experiment. Anti-CD40 mAb mediated higher levels of inhibition of IP-10 compared with anti-CD40L mAb. These results demonstrate that tumor Ag can activate IP-10 secretion in T cells of both healthy donors and breast cancer patients. This activation is dependent on MHC-I presentation, requires B7-CD28 and CD40-CD40L interactions, and it is potentiated by IL-12 and helper peptides. Thus, CD40L provides an activating signal for IP-10 induction; however, this signal appears not to be sufficient by itself to control IP-10 as it does IFN-γ.

DISCUSSION

We provide evidence that IP-10 is rapidly induced in the PBMC of healthy donors and in CD8$^+$ cells on stimulation with the HER-2 peptide E75. Induction of IP-10 by E75 can be detected 24 h after Ag contact and sometimes as early as 6 h (B.W. Anderson and T.V. Lee, unpublished observations). The implications of these findings are twofold: (1) E75 can rapidly stimulate T cells, specifically CD8$^+$ cells, to induce IP-10, and (2) IP-10 can be induced by IL-12 within 48 h in the absence of its exogenous inducer IFN-γ and of detectable levels of endogenous IFN-γ. Although the second point does not exclude the possibility that small levels of endogenous IFN-γ, together with other cytokines, may synergize with Ag in IP-10 induction, our results show that the levels of exogenous IFN-γ required to induce IP-10 in the same cells are considerably higher than the levels of IFN-γ detected at stimulation with E75 alone or together with IL-12. Furthermore, on a molar basis, the amount of IP-10 induced by E75 + IL-12 was significantly higher than the amount of IFN-γ.

Thus, it appears that tumor Ag, such as E75, can rapidly me-

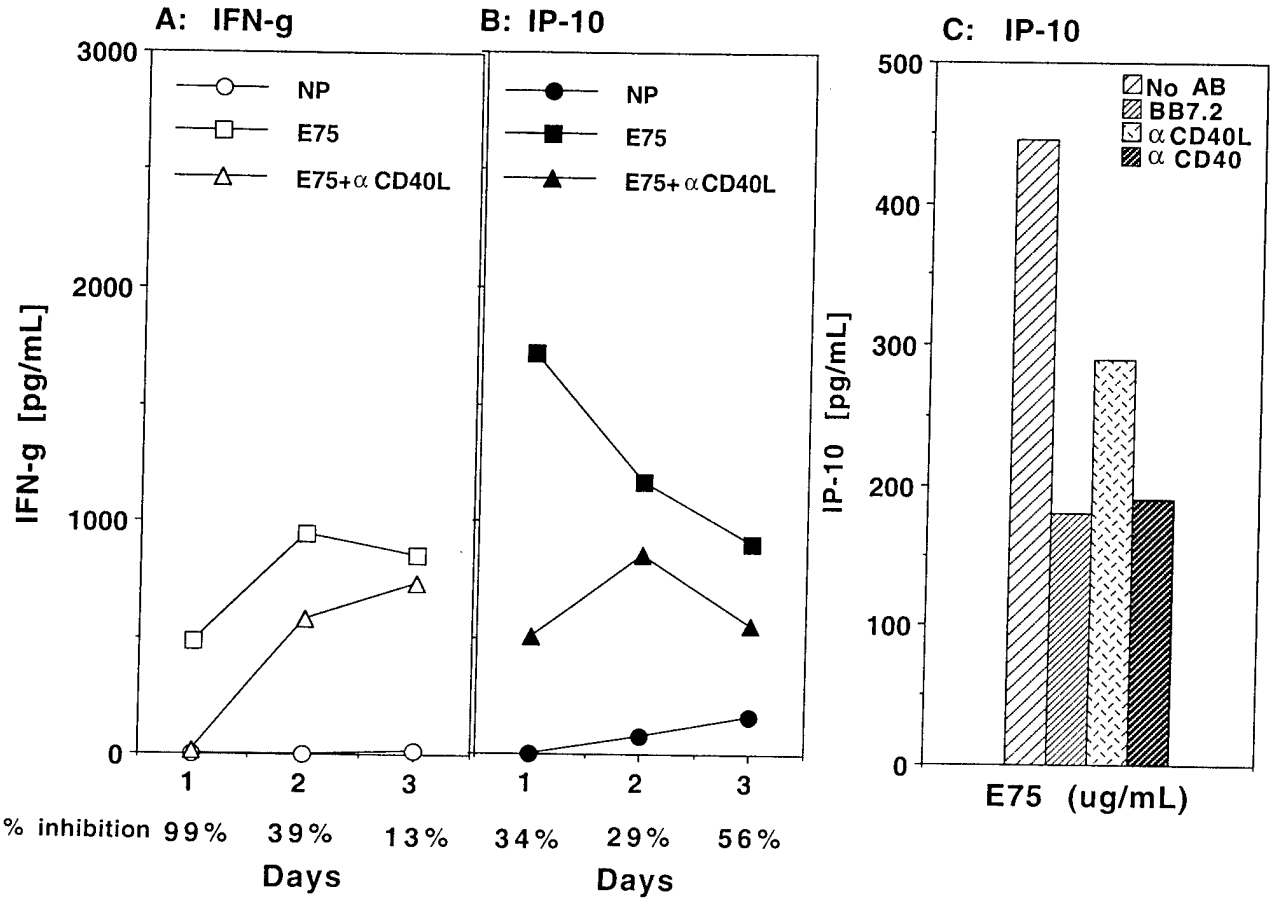

FIG. 6. Induction of IP-10 requires CD40L-CD40 interactions, and it is restricted by HLA-A2. Anti-CD40 and anti-CD40L were used at 5 ng/ml, according to the manufacturer's instructions, and were added to the cultures immediately after addition of responders. Percent specific inhibition was calculated by dividing the amount of IFN-γ or IP-10 secreted by E75 alone minus the amount of IFN-γ or IP-10 secreted by E75 with anti-CD40L by the amount of IFN-γ or IP-10 secreted by E75 alone. E75 was used at 100 μg/ml (**A,B**) and 10 μg/ml (**C**). **A,B** versus **C**, results from different experiments.

diate IP-10 induction. E75 effects were augmented by IL-12, suggesting a relationship between the ability of the Ag to activate APC, that is, DC, to induce IL-12 and the reciprocal effects of Ag + IL-12 in T cell activation. However, the role of the Ag appears critical in this process, as IP-10 production was modulated by E75 variants and enhanced by simultaneous stimulation with a helper HER-2 peptide.

To clarify the requirements for IP-10 induction by Ag, we performed Ab inhibition experiments. IP-10 induction was essentially abolished by blocking B7.1, suggesting a requirement for CD28 costimulation for IP-10 induction and raising the possibility that the responders may be naive or resting T cells. A similar effect was observed with anti-HLA-A2 mAb, indicating that Ag recognition in the appropriate context is a requirement for IP-10 induction. Of interest, although anti-CD40L completely inhibited IFN-γ induction, it was less effective in inhibiting IP-10 induction by Ag. This was confirmed in several independent experiments. Similarly, anti-CD40 at the same concentration failed to completely inhibit IP-10 production. The CD40-CD40L pathway is the main induction pathway of IL-12 in monocytes/DC in interaction with T cells.[26,27] Similarly, Ag and IL-12 synergize in IFN-γ induction.[28] However, IP-10 induction appears not to be exclusively controlled by this pathway. The reasons for this functional dichotomy are still unknown. CD40L is induced in T cells by Ag activation, with costimulatory and adhesion molecules providing a stabilizing role.[29] IL-12 also synergizes with Ag in upregulating CD40L expression on human T cells.[30] Stimulation of isolated $CD8^+$ cells with E75 presented on autologous DC induced IL-12 in DC. These levels were below the IL-12 levels required to costimulate IFN-γ in response to E75 (T.V. Lee et al., unpublished observations). Thus, one possibility is that the same low levels of IL-12 may be sufficient for costimulation of induction of IP-10 in response to E75, but by themselves they are insufficient for IFN-γ induction. Therefore, activation of chemokine induction may be more sensitive to Ag stimulation than to cytokine IFN-γ and IL-12 stimulation. Another possibility is that in lymphocytes, IP-10 induction may not be dependent on IFN-γ but may involve another signaling pathway. IP-10 may be induced at Ag stimulation by TCR-mediated signals together with CD28 costimulation, and the CD40L pathway may serve as a second amplifying signal for IP-10 when IL-12 and IFN-γ are

present. This may explain why anti-CD40L, although completely inhibiting IFN-γ induction, had a more limited inhibitory effect on IP-10 induction.

Transcription of the IP-10 gene in different cell types requires (IFN)-stimulated response element (ISRE) and κB sites.[31] ISRE binding factors that mediate transcriptional activation as well as repression have been reported.[11,31–33] The κB motif has been shown to be an essential *cis*-acting regulatory element for cytokine gene expression in a variety of cell types.[33] In fibroblasts, IFN-γ and TNF-α synergized in IP-10 induction. The synergy between IFN-γ and TNF-α was mediated by independent activation of ISRE and κB binding activity by each cytokine.[31] Thus, the synergy between Ag and IFN-γ/IL-12 may follow a similar pathway in lymphocytes, with the Ag activating certain κB family members and IFN-γ/IL-12 activating ISRE. Alternatively, TCR stimulation may upregulate IL-12Rβ2.[28] IL-12Rβ2, together with the constitutively expressed IL-12Rβ1, form the high-affinity IL-12R, which can signal Stat3/4 at low levels of IL-12.[28] Furthermore, it is possible that IP-10 may be able to activate Stat directly by signaling through its receptor CXCR3 without requiring Jak association, as recently demonstrated for RANTES and macrophage inflammatory protein-1α (MIP-1α).[34]

This may explain why IP-10 can be detected at stimulation with low concentrations of Ag even in the absence of exogenous IFN-γ. Stimulation with Ag may initiate a positive feedback mechanism in which Ag plus small amounts of endogenous IL-12 and IP-10 activate Stat1:3, leading to induction of small levels of IFN-γ. IFN-γ in turn can further amplify IP-10 induction. In fact, the ability of IP-10 to augment IFN-γ secretion in lymphocytes in response to mitogens or environmental Ag has been reported recently.[35] Thus, IP-10 induction may be initially mediated/initiated by Ag activation and may be further amplified by cytokines. In support of this possibility, it has been reported that stimulation of $CD8^+$ cells with nonspecific activators (PMA + ionomycin) or anti-CD3 induced a wide range of chemokines, such as RANTES, MIP-α, and IL-8.[36] The amounts of MIP-α and IL-8 secreted by $CD8^+CD45R0^+$ cells, which represent activated/memory cells, were significantly higher than the amounts secreted by $CD4^+$ cells.[36] Similarly, $CD8^+$ CTL lines secreted MIP-α, MIP-β, IL-16, and IP-10 at specific recognition of the proteolipid antigen[3,19] without requiring exogenous IFN-γ. In a different system, induction of MIP-α by anti-CD3 required B7-CD28 interactions, although this costimulation could not be substituted by IL-2.[37] This suggests that at least for some chemokines, stimulation through TCR + CD28 may induce chemokine gene transcription directly and not indirectly as a consequence of activation of cytokine gene expression. Indeed, it was shown recently that IP-10 inhibited IL-2-induced proliferation.[35]

Although quantitative and qualitative comparisons between the amounts of chemoattractant cytokines (CC) and CXC chemokines induced by Ag have not been established, these results suggest a balance and a possible regulatory role of chemokines in one group toward the other. CXCR3, the IP-10 and MIG receptor, was found to be expressed at high levels on Th0 and Th1 cells but at low levels on Th2 cells. In contrast, Th2 cells that expressed CCR3 and CCR4 responded to eotoxin.[4] In support of this possibility, it was shown recently that CXCR3 also has considerable affinity for CC, particularly the CCR3 ligands eotaxin and monocyte chemoattractant protein-4 (MCP-4). Binding of CC to CXCR3 blocked IP-10-mediated receptor activation, suggesting that chemokines of different subfamilies may act as natural antagonists for other families.[38]

Thus, the ability of chemokines to activate polarized populations of T cells and to induce polarization of T cell subsets may be important in modulating the immune response to gynecologic tumors. Stimulation with tumor Ag together with IL-12 may provide initial sensitization by chemokines for DC recruitment[39,40] and initiation of inflammation, and secretion of non-ELR-CXC chemokines by $CD8^+$ cells before IFN-γ may initiate migration of $CD4^+$ cell to tumor site and type 1/Th1 polarization.[41] Preliminary data in our laboratory show that IP-10 induction is not directly affected by IL-10. It remains to be seen how tumor Ag activation mediates induction of chemokines with agonistic and antagonistic effects toward each other and how the balance of chemokine response translates into further cytokine responses that may promote or inhibit tumor growth. The results in this report may be helpful for understanding the mechanisms of cellular immunity to tumors and of pathways to induce effective antitumor responses.

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REFERENCES

1. ABE, M., KONDO, T., XU, H., and FAIRCHILD, R.L. (1996). Interferon-γ inducible protein (IP-10) expression is mediated by $CD8^+$ T cells and is regulated by $CD4^+$ T cells during the elicitation of contact hypersensitivity. J. Invest. Dermatol. **107,** 360–366.
2. WARD, S.G., BACON, K., and WESTWICK, J. (1998). Chemokines and T lymphocytes: More than an attraction. Immunity, 9: 1–11, 1998.
3. BIDDISON, W.E., CRUIKSHANK, W. W., CENTER, D. M., PELFREY, C. M., TAUB, D. D., and TURNER, R. V. (1998). $CD8^+$ myelin peptide-specific T cells can chemoattract $CD4^+$ myelin peptide-specific T cells: importance of IFN-inducible protein 10. J. Immunol. **160,** 444–448.
4. SALLUSTO, F., LENIG, D., MACKAY, C.R., and LANZAVECCHIA, A. (1998). Flexible programs of chemokine receptor expression on human polarized T helper 1 and 2 lymphocytes. J. Exp. Med. **187,** 875–883.
5. TANNENBAUM, C. S., TUBBS, R., ARMSTRONG, D., FINKE, J. H., BUKOWSKI, R. M., and HAMILTON, T.A. (1998). The CXC chemokines IP-10 and MIG are necessary for IL-12-mediated regression of the mouse RENCA tumor. J. Immunol. **161,** 927–932.
6. ARENBERG, D. A., KUNKEL, S. L., POLVERINI, P. J., MORRIS, S. B., BURDICK, M. D., GLASS, M. C., TAUB, D. T., IANNETTONI, M. D., WHYTE, R. I., and STREITER, R. M. (1996). Interferon-γ-inducible protein 10 (IP-10) is an angiostatic factor than inhibits human non-small cell lung cancer (NSCLC) tumorigenesis and spontaneous metastases. J. Exp. Med. **184,** 981–992.
7. LUSTER, A.D., GREENBERG, S. M., and LEDER, P. (1995). The IP-10 chemokine binds to a specific cell surface heparan sulfate

site shared with platelet factor 4 and inhibits endothelial cell proliferation. J. Exp. Med. **182,** 219–231.

8. DIAS, S., THOMAS, H., and BALKWILL, F. (1998). Multiple molecular and cellular changes associated with tumour stasis and regression during IL-12 therapy of a murine breast cancer model. Int. J. Cancer **72,** 151–157.
9. SGADARI, C., ANGIOLILLO, A.L., and TOSATO, G. (1996). Inhibition of angiogenesis by interleukin-12 is mediated by the interferon-inducible protein 10. Blood **87,** 3877–3882.
10. OHMORI, Y., SCHREIBER, R.D., and HAMILTON, T.A. (1997). Synergy between interferon-γ and tumor necrosis factor-α in transcriptional activation is mediated by cooperation between signal transducer and activator of transcription 1 and nuclear factor κB. J. Biol. Chem. **272,** 14899–14907.
11. LUSTER, A.D., and RAVETCH, J.V. (1987). Genomic characterization of a gamma-interferon-inducible gene (IP-10) and identification of an interferon-inducible hypersensitive site. Mol. Cell. Biol. **7,** 3723–3731.
12. TOMURA, K., and NARUMI, S. (1999). Differential induction of interferon (IFN)-inducible protein 10 following differentiation of a monoctye, macrophage cell lineage is related to the changes of nuclear proteins bound to IFN stimulus response element and kappaB sites. Int. J. Mol. Med. **3,** 477–484.
13. KOPYDLOWSKI, K.M., SALKOWSKI, C. A., CODY, M.J., VAN ROOIJEN, N., MAJOR, J., HAMILTON, T. A., and VOGEL, S. N. (1999). Regulation of macrophage chemokine expression by lipopolysaccharide *in vitro* and *in vivo*. J. Immunol. **163,** 1537–1544.
14. COUGHLIN, C. M., SALHANY, K. E., WYSOCKA, M., ARUGA, E., KURZAWA, H., CHANG, A.E., HUNTER, C. A., FOX, J. C., TRINCHIERI, G., and LEE, W. M. F. (1998). Interleukin-12 and interleukin-18 synergistically induce murine tumor regression which involves inhibition of angiogenesis. J. Clin. Invest. **101,** 1441–1452.
15. COUGHLIN, C. M., SALHANY, K. E., GEE, M. S., LATEMPLE, D. C., KOTENKO, S., MA, X., GRI, G., WYSOCK, M., KIM, J. E., LIU, L., LIAO, F., FARBER, J. M., PESTKA, S., TRINCHIERI, G., and LEE, W. M. (1998). Tumor cell responses to IFNγ affect tumorigenicity and response to IL-12 therapy and antiangiogenesis. Immunity **9,** 25–34.
16. BARBULESCU, K., BECKER, C., SCHLAAK, J. F., SCHMITT, E., ZUM BUSCHENFELDE, K. M., and NEURATH, M. F. (1998). Cutting edge: IL-12 and IL-18 differentially regulate the transcriptional activity of the human IFN-γ promoter in primary CD4$^+$ T lymphocytes. J. Immunol. **160,** 3642–3647.
17. KOBLISH, H. K., HUNTER, C. A., WYSOCKA, M., TRINCHIERI, G., and LEE, W.M.F. (1998). Immune suppression by recombinant interleukin (rIL)-12 involves interferon γ induction of nitric oxide synthase 2 (iNOS) activity: inhibitors of NO generation reveal the extent of rIL-12 vaccine adjuvant effect. J. Exp. Med. **188,** 1603–1610.
18. LUSTER, A. D., and LEDER, P. (1993). IP-10, a C-X-C-chemokine, elicits a potent thymus-dependent antitumor response *in vivo*. J. Exp. Med. **178,** 1057–1065.
19. BIDDISON, W. E., TAUB, D. D., CRUIKSHANK, W. W., CENTER, D. M., CONNOR, E. W., and HONMA, K. (1997). Chemokine and matrix metalloproteinase secretion by myelin proteolipid protein-specific CD8$^+$ T cells. J. Immunol. **158,** 3046–3053.
20. BRADLEY, L. M., ASENSIO, V. C., SCHIOETZ, L. K., HARBERTSON, J., KRAHL, T., PATSTONE, G., WOOLF, N., CAMPBELL, I. L., and SARVETNICK, N. (1999). Islet-specific Th1, but not Th2, cells secrete multiple chemokines and promote rapid induction of autoimmune diabetes. J. Immunol. **162,** 2511–2520.
21. FISK, B., BLEVINS, T.L., WHARTON, J.T., and IOANNIDES, C.G. (1995). Identification of an immunodominant peptide of HER-2/neu proto-oncogene recognized by ovarian tumor-specific cytotoxic T lymphocyte lines. J. Exp. Med. **181,** 2109–2117.
22. HUDSON, J.M., CASTILLEJA, A., MURRAY, J.L., HONDA, T., KUDELKA, A., SINGLETARY, E., WHARTON, J. T., and IOANNIDES, C. G. (1998). Growth and antigen recognition of tumor-infiltrating lymphocytes from human breast cancer. J. Interferon Cytokine Res. **18,** 529–536.
23. RONGCUN, Y., SALAZAR-ONFRAY, F., CHARO, J., MALMBERG, K. J., EVRIN, K., MAES, H., KONO, K., HISING, C., PETERSSON, M., LARSSON, O., LAN, L., APPELLA, E., SETTE, A., CELIS, E., and KIESSLING, R. (1999). Identification of new HER2/neu–derived peptide epitopes that can elicit specific CTL against autologous and allogeneic carcinomas and melanomas. J. Immunol. **163,** 1037–1044.
24. TUTTLE, T.M., ANDERSON, B.W., THOMPSON, W.E., LEE, J.E., GRABSTEIN, K.H., SAHIN, A., WHARTON, J.T., IOANNIDES, C.G., and MURRAY, J.E. (1998). Proliferative and cytokine responses to MHC-class II associated HER-2 peptides in breast cancer patients. Clin. Cancer Res. **4,** 2015–2024.
25. SALLUSTO, F., and LANZAVECCHIA, A. (1994). Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor-α. J. Med. Exp. **179,** 1109–1118.
26. DEKRUYFF, R.H., GIENI, R.S., and UMETSU, D.T. (1997). Antigen-driven but not lipopolysaccharide-driven IL-12 production in macrophages requires triggering of CD40. J. Immunol. **158,** 359–366.
27. McDYER, J. F., GOLETZ, T. J., THOMAS, E., JUNE, C. H., and SEDER, R. A. (1998). CD40 ligand/CD40 stimulation regulates the production of IFN-γ from human peripheral blood mononuclear cells in an IL-12 and/or CD28-dependent manner. J. Immunol. **160,** 1701–1707.
28. GATELY, M. K., RENZETTI, L. M., MAGRAM, J., STERN, A., ADORINI, L., GUBLER, U., and PRESKY, D. H. (1998). The interleukin-12/interleukin-12-receptor system: role in normal and pathologic immune responses. Annu. Rev. Immunol. **16,** 495–521.
29. JAISWAL, A., I., DUBEY, C., SWAIN, S. L., and CROFT, M. (1995). Regulation of CD40 ligand expression on naive CD4 T cells: a role for TCR but not co-stimulatory signals. Int. Immunol. **8,** 275–285.
30. PENG, X., REMACLE, J. E., KASRAN, A., HUYLEBROECK, D., and CUEPPENS, J.L. (1998). IL-12 up-regulates CD40 ligand (CD154) expression on human T cells. J. Immunol. **160,** 1166–1172.
31. OHMORI, Y., and HAMILTON, T.A. (1995). The intereferon-stimulated response element and a κB site mediate synergistic induction of murine IP-10 gene transcription by IFN-γ and TNF-α. J. Immunol. **154,** 5235–5244.
32. CHENG, G., NAZAR, A., SHIN, H.S., VANGURI, P., and SHIN, M.L. (1998). IP-10 gene transcription by virus in astrocytes requires cooperation of ISRE with adjacent κB site but not IRF-1 or viral transcription. J. Interferon Cyokine Res. **18,** 987–997.
33. XIA, Y., PAUZA, M. E., FENG, L., and LØ, D. (1997). RelB regulation of chemokine expression modulates local inflammation. Am. J. Pathol. **151,** 375–387.
34. WONG, M., and FISH, E. N. (1998). RANTES and MIP-1 activate Stats in T cells. J. Biol. Chem. **273,** 309–314.
35. GANGUR, V., SIMONS, F. E. R., and HAYGLASS, K. T. (1998). Human IP-10 selectively promotes dominance of polyclonally activated and environmental antigen-driven IFN-γ over IL-4 responses. FASEB J. **12,** 705–713.
36. CONLON, K., LLYOD, A., CHATTOPADHYAY, U., LUKACS, N., KUNKEL, S., SCHALL, T., TAUB, D., MORIMOTO, C., OS-

BORNE, J., OPPENHEIM, J., YOUNG, H., KELVIN, D., and ORTALDO, J. (1995). $CD8^+$ and $CD45RA^+$ human peripheral blood lymphocytes and potent sources of macrophage inflammatory protein 1α, interleukin-8 and RANTES. Eur. J. Immunol. **25,** 751–756.

37. HEROLD, K. C., LU, J., RULIFSON, I., VEZYS, V., TAUB, D., GRUSBY, M. J., and BLUESTONE, J. A. (1997). Regulation of C-C chemokine production by murine T cells by CD28/B7 costimulation. J. Immunol. **159,** 4150–4153.
38. WENG, Y., SICILIANO, S. J., WALDBURGER, K. E., SIROTINA-MEISHER, A., STARUCH, M. J., DAUGHERTY, B. L., GOULD, S. L., SPRINGER, M. S., and DEMARTINO, J. A. (1998). Binding and functional properties of recombinant and endogenous CXCR3 chemokine receptors. J. Biol. Chem. **273,** 18288–18291.
39. SALLUSTO, F., and LANZAVECCHIA, A. (1999). Mobilizing dendritic cells for tolerance, priming, and chronic inflammation. J. Exp. Med. **189,** 611–614.
40. DE SAINT-VIS, B., FUGIER-VIVIER, I., MASSACRIER, C., GAILLARD, C., VANBERVLIET, B., AIT-YAHIA, S., BANCHEREAU, J., LIU, Y.-J., LEBECQUE, S., and CAUX, C. (1998). The cytokine profile expressed by human dendritic cells is dependent on cell subtype and mode of activation. J. Immunol. **160,** 1666–1676.
41. SARRIS, A.H., ESGLEYES-RIBOT, T., CROW, M., BROXMEYER, H.E., KARASAVVAS, N., PUGH, W., GROSSMAN, D., DEISSEROTH, A., and DUVIC, M. (1995). Cytokine loops involving interferon-gamma and IP-10, a cytokine chemotactic for $CD4^+$ lymphocytes: an explanation for the epidermotropism of cutaneous T-cell lymphoma? Blood **86,** 651–658.

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INDUCTION OF DETERMINANT SPREADING AND OF TH1 RESPONSES BY *IN VITRO* STIMULATION WITH HER-2 PEPTIDES

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Running title: Th1 cryptic determinants in HER-2

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Abstract

Immunization with tumor antigens induce cellular and humoral immune responses. These responses by T cells are specific for defined eiptopes (determinants) in the molecule of the immunizing tumor Ag. Expension of such responses to self-Ag requires induction of autoimmunity to tumor. According with systems of autoimmune disease, expression of T cell autoimmunity is charaterized by diversification of responses from the inducer determinant to other responder (cryptic) determinants. Since similar strategies may be useful for therapy of human cancers, we investigated whether induction of response to a HER-2 peptide F7 (776-789) induces enhanced reactivity of other HER-2 peptides. We found that stimulation with F7 can expand a response to another epitope F13 (884-899) in both on ovarian cancer patient with progressive disease and a healthy donor which shared HLA-DR11. This response was characterized mainly by increased IFN-γ secretion, and proliferation, but was not observed with another donor which shared HLA-DR14, and HLA-DQ5 with the patient. Since repeated vaccination with the same epitope may lead to decline of primary cell reactivity due to apoptosis spreading of the response to other epitopes in the tumor Ag may provide an approach for maintaining an inflammatory Th1 response during cancer vaccination.

Introduction

Studies during recent years identified tumor antigens (Ag) that are targets of tumor reactive CTL (reviewed by Boon and van der Bruggen (1). These Ag are self-proteins, which are capable to induce both a cellular response (mediated by $CD8^+$ or $CD4^+$ cells) and/or a humoral response. Recent experimental findings support the concept that CTL and $CD4^+$ cells recognizing tumor Ag can mediate tumor regression and justify the design and development of epitope directed cancer vaccines (2,3). For all tumor Ag the major issue that needs to be resolved is how to generate and optimize an immune response to the tumor. Although both CTL and helper-T cells have been identified to recognize peptide epitopes from proteins such as HER-2, gp100, MART-1, p53, etc. (4) and they could be easily expanded *in vitro* the disease progressed in patients suggesting that either the detected response is too weak to control cancer spread, or the response to tumor is silenced.

Repeated stimulation with the same CTL epitope enhanced anti-tumor CTL expansion with slow kinetics (5,7). Since the tumor environment is either tolerogenic or suppressive, CTL induction and expansion depends in many instances on exogenous cytokine help to promote CTL survival and maintenance of an inflammatory environment. Because of cytokine short half-life and side effects, help for longer intervals can be also provided by $CD4^+$ cells when they are activated by appropriate Ag. However, repeated vaccinations with the same $CD4^+$ helper epitope are accompanied by decline of primary T cell reactivity over time suggesting that reactive T cells may be eliminated by apoptosis (8). Thus, an alternative approach to repeated exogenous cytokine administration and repeated $CD4^+$ epitope application is to amplify the Th1 response by spreading (9,10) it to other epitopes which are endowed with Th1 cytokine secreting ability. Intramolecular epitope spreading under these circumstances should be more beneficial for an

antitumor response than intermolecular spreading because it activates responders to the tumor Ag of interest than to irrelevant Ag which may not be present at that time. This approach requires first identification of both the inducer and the amplified epitope. To address these questions we rationalized that if in a cancer patient responses to a number of HER-2 epitopes are detectable at primary stimulation during the disease-free period this will indicate an *in vivo* priming event by an epitope from HER-2 which is presented by APC. The decline in the responses to some epitopes during disease progression may point out to epitopes, which are no longer presented, as well as to tolerization of the existent responders. Analysis of the ability of these epitopes to induce diversification of responses to other epitopes with concomitant enhancement of Type 1 cytokine secretion, may define a stimulation sequence for induction of an inflammatory autoreactive response. We found that priming of PBMC from an ovarian cancer patient with the HER-2 peptide F7(776-789) induced diversification of this response to the HER-2 epitope F13 (884-899). The response to F7 in this patient was gradually lost during the stable disease period, while the response to F13 which was present during the same period was lost when the disease progressed. Analysis of specificity and restriction of this response in MHC-II matched donors indicated that this response was associated with HLA-DR11. These results support the hypothesis that intramolecular determinant spreading can be induced by HER-2 peptides and can lead to induction of autoimmunity to cancer antigens. The identified pattern of spreading can provide a basis for epitope selection for cancer vaccines development.

Materials and Methods

PBMC. Peripheral blood was collected from two healthy donors designated as donor 1 (HLA-A2, 23, B7.48, DR7, 11, DQ2.6), donor 2 (HLA-A11, 68, B51, 67, DR13, 14, DQ5, 6) and from an ovarian cancer patient, designated as patient 1 (HLA-A24,, 28, B35, W6, 70, CW3, 4, DR11, 14, DQ5). The HLA-typing for the healthy donors was performed in the Blood Bank of the M. D. Anderson Cancer Center, while the typing of the patient was performed using molecular methods. Peripheral blood mononuclear cells (PBMC) were separated by Ficoll-Hypaque and used for stimulation immediately after separation.

Antigens. The eight HER-2 peptides constructed by the Synthetic Antigen Laboratory of M. D. Anderson Cancer Center derived from the aminoacid sequence of the human HER-2. These peptides have been previously selected based on the computer program ANT.FIND.M. which was used for the prediction of candidate T cell epitopes on HER-2 protein (11). The sequences of peptides used in this study are as follows: D122(396-406): QLQVFETLEET, F12 (449-465) GISWLGLRSRELGSGL; G88 (450-463); ISWLGLRSRELGS; F7 (776-789) GSYVSRLLGICL; G89 (777-790); SPYVSRLLGICL; F13 (884-899) VPIKWMALESILRRRF; G90 (886-898); IKWMALESILRRR and F14 (474-487): TVPWDQ LFR NPHQA. The residues that are potential HLA-DR11 anchors are underlined. Because of the ability of peptides to bind on alternative registers, as well as of mutations in the MHC-class II binding pocket, motifs that predict specific binding to MHC-class II are not yet well defined. Most of these HER-2 peptides contain a minimum of two of the three major anchors reported for the major HLA-DR types (i.e. HLA-DR4, -DR1, -DR11). A larger panel of HER-2 peptides was previously tested for the ability to induce proliferation of PBMC from randomly selected healthy donors and ovarian cancer patients (12). Results of our previous study indicated that F7 and F13

induced proliferative responses of PBMC of healthy donors (57% and 62%, respectively) and cancer patients (24 and 21%, respectively) with higher frequency than the other HER-2 peptides tested. In contrast F12 was less frequently recognized (21% in healthy donors and 4% in cancer patients) (12). F7, and mainly his analog G89 (777-790) induced proliferation of PBMC from a large number of HLA-DR4+ breast cancer patients (20). The HER-2 intracellular (ICD) and extracellular domains (ECD) were gifts from Dr. Kenneth Grabstein, Corixa Corporation and were prepared as described (13).

T Cell Proliferation Assays. For characterization of T cell responses to HER-2 peptides, PBMC were plated into 24 well plates at a final concentration of 2 X 10^6/ml in complete RPMI medium supplemented with 5% human AB serum (12,13). Each peptide was added at a final concentration of 20 μg/ml. Five and six days later, equal volumes of cells were plated in tetraplicate in a 96 well plate, incubated with 1 μCi of tritiated thymidine (3H-Tdr) (Amersham) and counted as previously described (12,13). Results are expressed as stimulation index (S.I.) representing the ratio between the mean c.p.m. of the cultures stimulated with peptide, and the mean c.p.m. of the cultures that have not been stimulated with peptide (N.P.). For *in vitro* expansion of T cell cultures, six days after the primary stimulation, IL-2 (Cetus) was added in each culture at a final concentration of 20 Cetus U/ml for four – five additional days. Afterwards, over the next five days, IL-2 was gradually removed from these cultures. For the last 48 h before restimulation, the lymphocytes were rested by being cultured in the absence of exogenous IL-2. Restimulated experiments were performed in same way as primary stimulation with the difference that APC were X-Ray irradiated (10,000 Rads) autologous PBMC.

Cytokine Assays. The ability of PBMC to release cytokines in response to Ag stimulation was determined by culturing PBMC either as unstimulated (NP) or stimulated with

the corresponding peptides. Supernatants were collected after 48 h and stored frozen at –20°C until assayed for cytokine levels. IFN-γ, IL-4 and IL-10 were measured by double sandwich ELISA using the corresponding kits provided by Biosource International (Camariyo, CA). The assays were calibrated with human recombinant IFN-γ, IL-4 and IL-10 to detect each cytokine in the range of 10-10,000 pg/ml.

Results

Stimulation of PBMC from an Ovarian Cancer Patient with HER-2 Peptide F7 Induced Intramolecular Spreading of Response to HER-2 Peptide F13. The responses of patient 1 to HER-2 peptides were tested five times over a period of twenty-one months. Initially, when the disease in this patient was stable, its PBMC responded to F7, borderline to F13 and F14, but not to HER-2 peptides D122 and F12. Thus, the responses of this patient to F7, F13, and F14 were considered specific. During the following 16 months of stable disease the responses to F7 and F14 gradually declined, but the patient maintained a borderline response to F13. The proliferative response to F13 declined when the disease progressed (**Figure 1**). This could be interpreted as selective tolerization or deletion by apoptosis of cells responding to F7, F14 and F13. Alternatively, it was possible that F7 and F14 were no longer presented by APC, while F13 continued to be presented during the period when the disease was stable. In a previous report (12) we showed that during the stable disease period, F13-primed cultures: (a) responded to F13 with better proliferation than F7-primed cultures to F7; (b) showed a weak proliferative response to F7. The ratio IFN-γ/IL-10 secreted from F13-primed cultures in response to polyclonal activators OKT3+TPA was higher than from F7-primed cultures. This suggested that either these epitopes were cross-reactive, or priming with F13 enhanced the ability of F7-reactive cells to respond to F7 (12). This also suggested that the *in vivo* and *in vitro* priming response to F7 was insufficient, in either quantitative or qualitative terms, to mediate polarization of the environment for a Th1 response. F7-reactive cells either secreted low levels of IFN-γ and high levels of IL-10, or higher levels of IFN-γ than IL-10 but because their numbers were smaller than of the responders to F13, the amount of IFN-γ was not sufficient to inhibit the high background of Th2 cytokines (12).

The fact that at the time of disease progression responses to both F7 and F13 were no longer detected raised the possibility to address the ability of F7 and F13 to reverse the tolerant (nonresponsive) state to each other, and to establish whether they can serve as inducer and amplifying epitope, respectively.

PBMC collected at 21 months and stimulated with F7, F13, F12 and control NP showed insignificant proliferative responses. These peptides induced low levels of IFN-γ at priming (NP = 37, F7 = 72, F12 (negative control) = 42, and F13 = 68 pg/ml), but the IL-10 levels were high in all cultures (>250 pg/ml). To address whether responses to F7 and F13 were enhanced at reactivation, the cultures primed with F7 and F13, were restimulated with F7 and F13 using no peptide (NP) as control. The results in **Figure 2A** show that F13-primed cells responded to F7 and F13, while F7-primed cells responded only to F13 with S.I. < 2.0, but with significantly higher cpm compared with control (NP) stimulated cultures. This small increase in proliferative responses in F7F7 compared with F7 NP was considered not significant. This suggested that although F7 and F13 reactive cells were present, their numbers were too small to show a significant increase in proliferation levels.

In contrast, analysis of IFN-γ and IL-4 levels showed that F7-primed cultures increased their IFN-γ response by two-fold at restimulation and responded to F7 with slightly higher levels of IFN-γ than to F13. F13-primed cultures also responded to F7 with slightly higher levels of IFN-γ than to F13. The IL-4 levels decreased in response to both F7 and F13 compared with F7 and F13 primed cultures, which were stimulated with APC only (NP). The IFN-γ/IL-4 ratios were similar in both cultures. The most dramatic effect of peptide restimulation was observed with regard IL-10 induction. The IL-10 levels in control cultures, not stimulated with peptide at both priming and restimulation, (NPNP) were high (234 pg/ml). Priming with F7 and F13

slightly decreased IL-10 levels to 190-200 pg/ml. Restimulation with F7 and F13 decreased IL-10 levels even further by 30-40% (**Figure 2A and 2B**). These results suggested that F7-primed and F13-primed cells from this patient enhanced their response at restimulation with the same peptides since they modulated their cytokine response but the numbers of specific responders were too low to detect significant changes in proliferation. The IFN-γ/IL-10 ratios indicated that at restimulation F7 induced a stronger Th-1 cytokine response than F13.

Since stimulation with F7 appeared to enhance the IFN-γ response to F13 we investigated whether these peptides induced spreading of response to each other. To address whether there is reciprocal spread of F7 and F13 proliferative responses, each of the F7 primed and F7 restimulated cultures, designated F7F7) and of F13 primed and F13 restimulated cultures (designated F13F13) were restimulated with F7 and F13. F7F7 stimulated cells showed significant proliferation in response to both F7 and F13 (**Figure 3A**). This expansion of the proliferative response was accompanied by a 2-3 fold increase in the IFN-γ levels (**Figure 3B**). In contrast, F13F13 stimulated cells showed a weaker increase in proliferative responses to either F7 or F13. S.I. > 2.0 of proliferative responses was not observed in F13 F13, cells stimulated by F7 and F13 but the cpm levels were still significantly higher than of F13F13 NP cultures (**Figure 3C**).

The IFN-γ response paralleled the proliferative responses. The IFN-γ response to F7 of both F7F7 and F13F13 cells was higher than the response to F13. In addition, the IFN-γ/IL-4 ratio in F7F7F13 stimulated cultures (**Figure 3B**) was almost double the IFN-γ/IL-4 ratio in F13F13F13 (**Figure 3D**) stimulated cultures. These results suggested that F7 had a stronger Th1 inducing effect than F13 in this system. These results indicated that priming and restimulation with F7 (F7F7) expanded a response to F13, but priming with F13 (F13F13) was less effective in

expanding the response to F7. The requirement for F7 for induction of spreading of the response was confirmed by the fact that F13 primed cultures restimulated with F7, i.e. (F13→F7), the S.I. at the third stimulation were significantly higher than 2.0 in response to F7 (F13F7F7=4.45) compared with control (F13F7NP=0.85) but were not higher in response to F13 (F13F7F13=1.42).

Spreading of Th1 responses by F7 and F13 in healthy donors. The results from stimulation with F7 and F13 in this patient indicated that F7 induced a Th1 response and increased the ability of F13 reactive cells to respond to F13. Since this patient expressed HLA-DR11 and HLA-DR14, this raised the question of the association of the response with one MHC-II product or both. HLA-DR11 (DRB1* 1101) is a split of DR5, while HLA-DR14 (DRB1* 1401) is a split of HLA-DR6. Thus, these class II antigens belong to distinct genetic and serologic groups Anchors for both major HLA-DR types are present in both peptides. Since additional amounts of blood could not be obtained from this patient, because its disease progressed, the specificity analysis was performed using T cells from two partially HLA-DR matched healthy donors. Donor 1 shared only DR11 with patient 1 while donor 2 shared DR14 and DQ5 but not DR11 with patient 1. Therefore if responses will be found in donor 1, but not in donor 2 this will suggest that these peptide induce determinant spreading in association with DR7, 11 and DQ6. Since DR11 is expressed by the ovarian cancer patient this will confirm DR11, without excluding DR7 and DQ2. Alternatively, if responses will be found in donor 2, this will point out to HLA-DR14 as presenting molecule without excluding others. This analysis also aimed to address whether, (a) T cells from healthy donors respond with a similar or a different pattern of

cytokines to F7 and F13 and, (b) F7 can induce determinant spreading in healthy donors *in vitro* or spreading required prior *in vivo* priming with the tumor Ag during disease.

To address these questions PBMC from donor A ($DR11^+$) were primed with control (NP) as well as with F7, F12 and F13. Positive responses of PBMC to priming with these peptides were borderline only for F13 (NP=816 ± 29 cpm; F7 = 779 ± 54 cpm, F12 = 893 ± 61 cpm, F13 = 1327 ± 97 cpm, F14 = 1007 ± 59 cpm. This S.I. indexes ranged between 0.97 (F7) and 1.63 (F13). Only the cpm values of F13 and F14 were significantly higher than of control NP but the overall values were low. IFN-γ was detected only in response to F13 and its levels were 70 pg/ml (data not shown). Each peptide primed culture was restimulated with NP, F7, and using F12 as a control. The results in **Figure 4A** and **4C** show that F7-primed cells responded to both F7 and F13, but with higher proliferation to F13 (S.I. > 4.5) than to F7 (S.I.>2.5). These responses were mediated by $CD4^+$ cells based on inhibition by anti-CD4 and anti-HLA-DR mAb (L243), but not by anti-CD8 (Table 1). In parallel, F13-primed cells also responded to F7 and F13 but with higher proliferation to F13 **(Fig. 4C)**. Although the proliferative responses of both F7 and F13 primed cells to restimulation with F13 were higher than to restimulation with F7, their IFN-γ response to F7 was higher than the response to F13. These results confirmed that F7 can induce determinant spreading as detected in patient 1. In this healthy donor, F13 could also induce determinant spreading. The specificity of this response for F7 and F13 is illustrated by the fact that proliferative responses to F12 were not observed while the increase in IFN-γ was minimal. Also, priming with F12 did not diversify the response to F7 and F13 (not shown). The patterns of IFN-γ and IL-4 secretion in response to F7 and F13 were similar in both F7 and F13 primed cultures. The main distinction between these cultures was that the overall levels of IFN-γ were lower in F7F7-stimulated cultures, than in F13F7-stimulated cultures. The levels of IL-4 in

response to F7F7 were borderline (10-20 pg/ml), which is within the low levels of sensitivity of the method **(Fig. 4B)**. The levels of IL-4 in response to F13 F13 stimulation were higher than in response to F7 F13 **(Fig. 4D vs 4B)**. Consequently, at similar IFN-γ levels, the IFN-γ/IL-4 ratios were almost two times higher in the cultures primed with F7 than in the cultures primed with F13. It should be mentioned that because the IL-4 levels in response to F7F7 and F13F7 were low and at the lower levels of sensitivity of the ELISA method (20-50 pg/ml) the differences in the IFN-γ/IL-4 ratios between these two groups may not accurately reflect higher Th1 activity in the F7F7 group compared with F13F7 group. In contrast with patient 1, the levels of IL-10 in both F7-primed cultures and in F13-primed cultures, were borderline (<10 pg/ml) regardless of the peptide used for restimulation.

To address whether the lower S.I. for F7→F7 and F13→F7 stimulated cells were due to the lower numbers of F7-reacting precursors than F13-reacting cells, the corresponding cultures were restimulated with F7 and F13. At the third stimulation, S.I. for F7F7 stimulated cells was higher than S.I. for F13F13 F13 stimulated cells, but the highest increase was observed where F7F7 cultures were stimulated with F13: F13→F13→F13 (S.I.=4.6) F7→F7→F7 (S.I. = 5.5) compared with F7→F7→F13 (S.I = 9.8). F7→F7→F13 cells secreted higher levels of IFN-γ than F7→F7→F7 cells (900 pg/ml, compared with 480 pg/ml). Thus, the shift from F7 to F13 at the third stimulation allowed both high proliferation and IFN-γ production by F13 responding cells. It should be mentioned that both F7-stimulated and F13 stimulated cells recognized HER-2 ICD protein (data not shown).

The priming and restimulation experiments were repeated with donor 2 (which shared DR14 and DQ5 with patient 1) and expressed DR13 and DQ6. F7, F12, and F13 could neither induce proliferation at priming nor spreading of responses at restimulation in donor 2 (Figure

5A-5D). The levels of IFN-γ at restimulation with F7 were also low compared with donor 1 or patient A. Since responses were not found at restimulation this suggested that F7, F13 (as well as F12) are not presented in association with DR13, DR14 and DQ5 and DQ6. These results indicated that induction of inflammatory response to HER-2 peptides F7 and F13 was associated with HLA-DR11 as shared antigen between patient and donor PBMC although DR.7 and DQ6 cannot be excluded.

Discussion

In this study we identified a HER-2 epitope which initiated determinant spreading and inflammatory cytokine response in a healthy donor and an ovarian cancer patient which shared HLA-DR11. This response appeared to be restricted by HLA-DR11 since two other healthy donors which were HLA-DR14 + and did not share HLA-DR11 with the patient either failed to respond at stimulation with these peptides, (Donor 3) or developed only a weak response. (Donor 2) characterized by weak IFN-γ induction and proliferation. The pattern of spreading of responses differed between the patient and HLA-DR11 matched healthy donor. In the patient, of the two candidate Th1 peptides (F7 and F13) only F7 induced the spreading of response to F13, while in the healthy donor both F7 and F13 induced response spreading to each other. Furthermore, in the ovarian cancer patient studied, the increase in IFN-γ production, and the concomitant decrease in IL-10 preceded the detected expansion of the proliferative response. In contrast, in the healthy donor, the levels of IL-10 were low and the increase in IFN-γ secretion paralleled the increase in proliferative response.

The spreading of the proliferative and cytokine response from F7 to F13 in the patient appeared to be mediated at least in part by the ability of F7 to modulate the cytokine profiles of the responders. The IFN-γ/IL-10 ratios at restimulation of F7 and F13-primed cells F7→F7, F7→F13, F13→F13, F13→F7 were 1.73, 1.33, 0.89 and 1.4 respectively. This suggested that F7 priming and restimulation modulated the response towards Th1 more than F13. In contrast, in the healthy donor, not only the levels of IFN-γ were high at restimulation, but the levels of IL-10 were borderline, thus facilitating the ability F13 to condition the F7-responders or the for a higher IFN-γ response to F7. It should be noted that in the healthy donor, induction of inflammatory and proliferative responses to F7 and F13 required only two stimulations, while in

the patient three stimulations were required to obtain the same effect. The background levels of IL-10 were several folds higher in the cancer patient than in the healthy donor, raising the possibility that IL-10 may have downregulated the proliferative responses to F7 and F13.

The reasons for the differences between F7 and F13 in inducing proliferation and IFN-γ are unclear. One possibility to be considered is that the frequency of F7-responding cells in both the donor and the patient is significantly lower than the frequency of F13-reponsive cells. In this case the increase in proliferation by F7 as measured by the thymidine incorporation will not be detected when the numbers of responders are low, but will be detected after they reached a significant percentage of the cells in culture. This possibility is supported by: (a) the fact that F7 responding cells proliferated better at the third stimulation in both healthy donors and patient compared with F13-responding cells and; (b) F7-primed cells responded with higher IFN-γ levels than F13-primed cells. This suggested that F7-responders recognized F7 with higher affinity than F13-responders since they secreted higher levels of IFN-γ.

The demonstration that HER-2 peptides can induce intramolecular spreading of inflammatory responses is of interest for cancer vaccine development. Introduction in vaccines of helper epitopes such as F7 can reactivate the F7-reactive cells to secrete IFN-γ, and enhance the ability of F13-reactive cells to respond. Furthermore, if the response to F7 is low and/or F7-responders are tolerized, another Th1 epitope can be used as priming, followed by F7 and F13 at the next round of vaccination. Likely candidates for this function are the Th1 epitope from influenza associated with HLA-DR4 (14) and the helper epitope PADRE, developed by Sette and collaborators (15). The use of defined epitopes from the same protein or of genes encoding defined epitopes should avoid induction of Th2 responses by other epitopes which may be presented simultaneously at competition with F7 and F13. Furthermore, the successive

application of Th1 epitopes in a vaccination protocol should avoid induction of apoptosis in responders by the repeated stimulation with the priming epitope. In support of this possibility Disis and collaborators recently reported that vaccination with HER-2 peptides induce both intra- and intermolecular spreading of responses in terms of proliferation.

The results also points to the fact that peptide vaccination should be more effective than whole protein in inducing type 1 response spreading, for several reasons: (a) Since the presentation of HER-2 epitope is still an unknown, vaccination with e.g. F7 followed by F13 should focus the response to Th1 epitopes and avoid presentation of HER-2 epitopes which may induce this Th2 response; (b) sequential peptide application may allow to control spread and terminate the response when side effects are observed; (c) The use of peptides or of the genes encoding these peptides allows to lower the amounts of Ag, or of the viral vector used for vaccination. Since on a molar basis, 100 fold more HER-2 protein is needed to reach the same concentration as F7/F13, this may assist therapies using non-replicating viruses where the amount of Ag is limited.

The relationship between disease progression and decrease in responses to F7 and F13 deserves further investigation. The gradual decrease in responses to F7 during the stable disease period may suggest that F7 is less presented over time, while F13 continued to be presented. The decrease in response to F13 may indicate either that F13 is no longer presented by APC to stimulate and maintain the pool of F13 responders, or that suppression of F13 responding cells by Th2 cytokines secreted by the tumor, inhibited their ability to respond to F13. It is possible that F7 responding cells in this patient had a critical role in conditioning the environment or the APC for a Th1 response and were needed to sustain the ability of F13-specific cells to respond. In support of this possibility Steinman and collaborators postulated that

a small number of autoreactive cells in an infiltrate can control the responses of other cells to self-Ag (17). Determinant spreading has been described as an essential component of the progressive course of autoimmune disease (16,22). Broadening of the response to additional determinants to the primary epitope, led to more advanced autoimmune disease because of the diversified autoreactive pool of T cells. The fact that stimulation with HER-2 peptides can induce determinant spreading and Th1 responses may have important implications for development tumor immunity and of cancer vaccines development. While in autoimmune diseases characterized by type 1 response prevention of spreading is a main objective for therapy (19), in cancer, induction of spreading of a type 1 response, may be desired to induce a response against tumors. Identification of inducer and amplifying epitopes and may be of interest and for other tumor Ag recognized by CD4+ cells (20-23) provide a modality to control the expansion of a therapeutic response.

Figure Legends

Figure 1. Diagram of plotting proliferative responses over time of PBMC from patient 1, stimulated with each of the HER-2 peptides. S.I. were calculated by comparing the cpm proliferation in each independently performed experiment as described in Materials and Methods. SD indicates stable disease, P.D. indicates progressive disease. The average cpm for the experiments performed on months 0 and 19 were: Month 0: NP=595±59; F7=2050±101 F13=1498±71; D122=560±64; F14=1004±79; F7, F13 and F14-induced cpm all significantly higher than control no peptide (NP) stimulated (595±59). Month 6: NP=744±43; F7=943±83; F13=1664±119; F14=623±58. F13-induced cpm were significantly higher than NP and F7 induced cpm * = ($p<0.05$). The cpm values for the experiments performed on months 5 and 19 were in the same range as in the months 0 and 16.

Figure 2. Proliferative and cytokine responses of F7-primed and F13-primed PBMC from patient 1, collected on month 21 to F7 and F13. (A) F7 primed; (B) F13 primed cells. 4×10^4 PBMC primed with F7 or F13 were restimulated with 10^5 autologous indicated PBMC. The concentration of exogenous pulsed peptide was 20 μg/ml. The experiment was performed in triplicate. (B) Cpm from restimulation of F7-primed cells with F13, of F13-primed cells with F13, and F7 are significantly higher (* = ($p<0.05$) from cpm stimulation for NP, but stimulation indexes are < 2.0 i.e. F7NP=1631±96; F7F7=2194±187; F7F13=2936±140, and F13NP=1056±70; F13F7=1754±129; F13F13=1740±99. Numbers above open columns indicate IFN-γ/IL-4 ratios.

Figure 3A, B, C, D. Proliferative (A,C) and cytokine (B,D) responses of PBMC from patient 1 at third stimulation with F7 and F13. F7-F7 and F13-F13 indicates that responders were stimulated twice with F7 and F13. Cpm and S.I. for F7-and F13-stimulated, F13F13 cultures were significantly higher from cpm for NP-stimulated F13F13 cultures: F13F13 NP=1427 ± 59, F13F13F7=2995 ± 112 (S.I.=2.1) F13F13F13=2553 ± 61 (S.I.=1.80) (* = $p<0.05$). Experimental conditions as described in Fig. 2.

Figure 4A, C, D. Proliferative (A,C) and cytokine (B,D) responses of PBMC from healthy donor 1 at restimulation with F7 and F13. F12 was used as a negative control peptide. Experimental conditions are as described in Figure 2.

Figure 5A, B. Proliferative (A) and cytokine responses (B) of PBMC from donor 2 at restimulation with F7 and F13. S.I. and IFN-γ levels induced by F7, F12 and F13 are not significantly different from values obtained with NP. One of the two independently performed experiments with similar results from donor is shown. Experimental conditions as described in Fig. 2.

Table I. MHC-recognition by F7F7 stimulated cells

MAb	F7-stimulated cells (cpm)	Peptide/S.I.	% Inhibition
-	2019 ± 60	-/1.0	0
-	5242 ± 57	F7/2.6	0
αCD3	2472 ± 82	F7/1.22	87
αCD4	3007 ± 125	F7/1.49	70
αCD8	4990 ± 118	F7/2.47	7
αMHC-I(W6/32)	4462 ± 97	F7/2.21	24
αMHC-II (L243)	2836 ± 75	F7/1.40	75

Donor 1 PBMC were primed with F7 for 5 days, followed by expansion in low concentration (20-40 IU/ml) of IL-2 for the following two weeks. Afterwards, the cells were rested for two days by removing IL-2. F7 primed T-cells from this donor were stimulated with F7 pulsed at 20 μg/ml on 10^5 autologous irradiated (10,000 rad) autologous PBMC. 20,000 responders were added in each well in the presence and absence of the indicated monoclonal antibodies. The experiment was performed in tetraplicate. ^{3}H-Tdr was added on day 4, for the last 16 h of incubation. % inhibition was calculated by subtracting the cpm values for each mAb treated cultures and dividing the resulting cpm by the cpm values of the positive control (cultures stimulated with F7) minus the negative control cpm values.

References

1. Boon, T. and van der Bruggen, P. Human tumor antigens recognized by T lymphocytes. J. Exp. Med., 183:725-729, 1996.

2. Surman, D.R., Dudley, M.E., Overwijk, W.W. and Restifo, N.P. Cutting Edge: $CD4^+$ T cell control of $CD8^+$ T cell reactivity to a model tumor antigen. J. Immunol., 164:562-565, 2000.

3. Topalian, S.L. MHC class II restricted tumor antigens and the role of CD4+ T cells in cancer immunotherapy. Curr. Opin. Immunol., 6:741-745, 1999.

4. Wang, R.F. and Rosenberg, S.A. Human tumor antigens for cancer vaccine development. Immunol. Review, 170:85-100, 1999.

5. Marincola, F.M., Rivoltini, L., Salgaller, M.L., Player, M. and Rosenberg, S.A. Differential anti-MART-1/MelanA CTL ability in peripheral blood of HLA-A2 melanoma patients in comparison to healthy donors. Evidence of *in vivo* priming by tumor cells. J. Immunother. 19:266-277, 1996.

6. Perez-Diez, A., Butterfield, L.H., Li, L., Chakraborty, N.G., Economou, J.S. and Mukherji, B. Generation of CD8+ and CD4+ T-cell response to dendritic cells genetically engineered to express the MART-1/Melan-A-gene. Cancer Res. 58:5305-5309, 1998.

7. Anichini, A., Molla, A., Mortarini, R., Tragni, G., Bersani, I., DiNicola, M., Gianni, A.M., Pilotti, S., Dunbar, R, Cerundolo, V. and Parmiani G. An expanded peripheral T cell population to a cytotoxic T lymphocyte (CTL)-defined, melanocyte-specific antigen in metastatic melanoma patients impacts on generation of peptide-specific CTLs but does not overcome tumor escape from immune surveillance in metastatic lesions. J. Exp. Med., 190:651-6678, 1999.

8. Tuohy, V.K., Yu, M., Yin, L., Kawczak, J.A., Johnson, J.M., Mathisen, P.M., Weinstock-Guttman, B. and Kinkel, R.P. The epitope spreading cascade during progression of experimental autoimmune encephalomyelitis and multiple sclerosis. Immunol. Reviews, 164:93-100, 1998.

9. Lehmann, P.V., Forsthuber, T., Miller, A. and Sercarz, E.E.Spreading of T-cell autoimmunity to cryptic determinants of an autoantigen. Nature, 358:156-157, 1992.

10. Kaufman, D.L., Clare-Salzler, M., Tian, J., Forsthuber, T., Ting, F.S.P., Robinson, R., Atkinson, M.A., Sercarz, E.E., Tobin, A.J. and Lehmann, P.V. Spontaneous loss of T-cell tolerance to glutamic acid decarboxylase in murine insulin-dependent diabetes. Nature, 366-69-71, 1993.

11. Ioannides, C.G., Ioannides, M.G. and O'Brian, C.A. T cell recognition of oncogene products. Mol. Carcinogen., 6:77-81, 1992.

12. Fisk, B., Hudson, J.M., Kavanagh, J., Wharton, J.T., Murray, J.L., Ioannides, C.G. and Kudelka, A.P. Existent proliferative responses of peripheral blood mononuclear cells from healthy donors and ovarian cancer patients to HER-2 peptides. Anticancer Res., 17:45-54, 1997.

13. Tuttle, T., Anderson, B.W., Thompson, W.E., Lee, J.E., Sahin, A., Smith, T.L., Grabstein, K.H., Wharton, J.T., Ioannides, C.G. and Murray, J.L. Proliferative and cytokine responses to class II HER-2/neu-associated peptides in breast cancer patients. Clin. Cancer Res., 4:2015-2024, 1998.

14. Marshall, K.W., Wilson, K.J., Liang, J., Woods, A., Zaller, D. and Rothbard, J.B. Prediction of peptide affinity to HLA-DRB1 *0401. J. Immunol., 154:5927-5933, 1995.

15. del Guercio, M.F., Alexander, J., Kubo, R.T., Arrhenius, T., Maewal, A., Appella, E., Hoffman, S.L., Jones, T., Valmori, D., Sakaguchi, K., Grey, H.M. and Sette, A. Potent immunogenic short linear peptide construcs composed of B cell eiptopes and Pan DR T helper epitopes (PADRE) for antibody responses *in vivo*. Vaccine, 15:441-448, 1997.

16. Disis, M.L., Grabstein, K.H., Sleath, P.R. and Cheever, M.A. Generation of immunity to the HER-2/*neu* oncogenic protein in patients with breast and ovarian cancer using a peptide-based vaccine. Clin. Cancer Res., 5:1289-1297, 1999.

17. Steinman, L. A few autoreactive cells in an autoimmune infiltrate control a vast population of nonspecific cells: A tale of smart bombs and the infantry. Proc. Natl. Acad. Sci. USA, 93:2253-2256, 1996.

18. Yu, M., Johnson, J.M. and Tuohy, V.K. A predictable sequential determinant spreading cascade invariably accompanies progression of experimental autoimmune encephalomyelitis: A basis for peptide-specific therapy after onset of clinical disease. J. Exp. Med., 183:1777-1788, 1996.

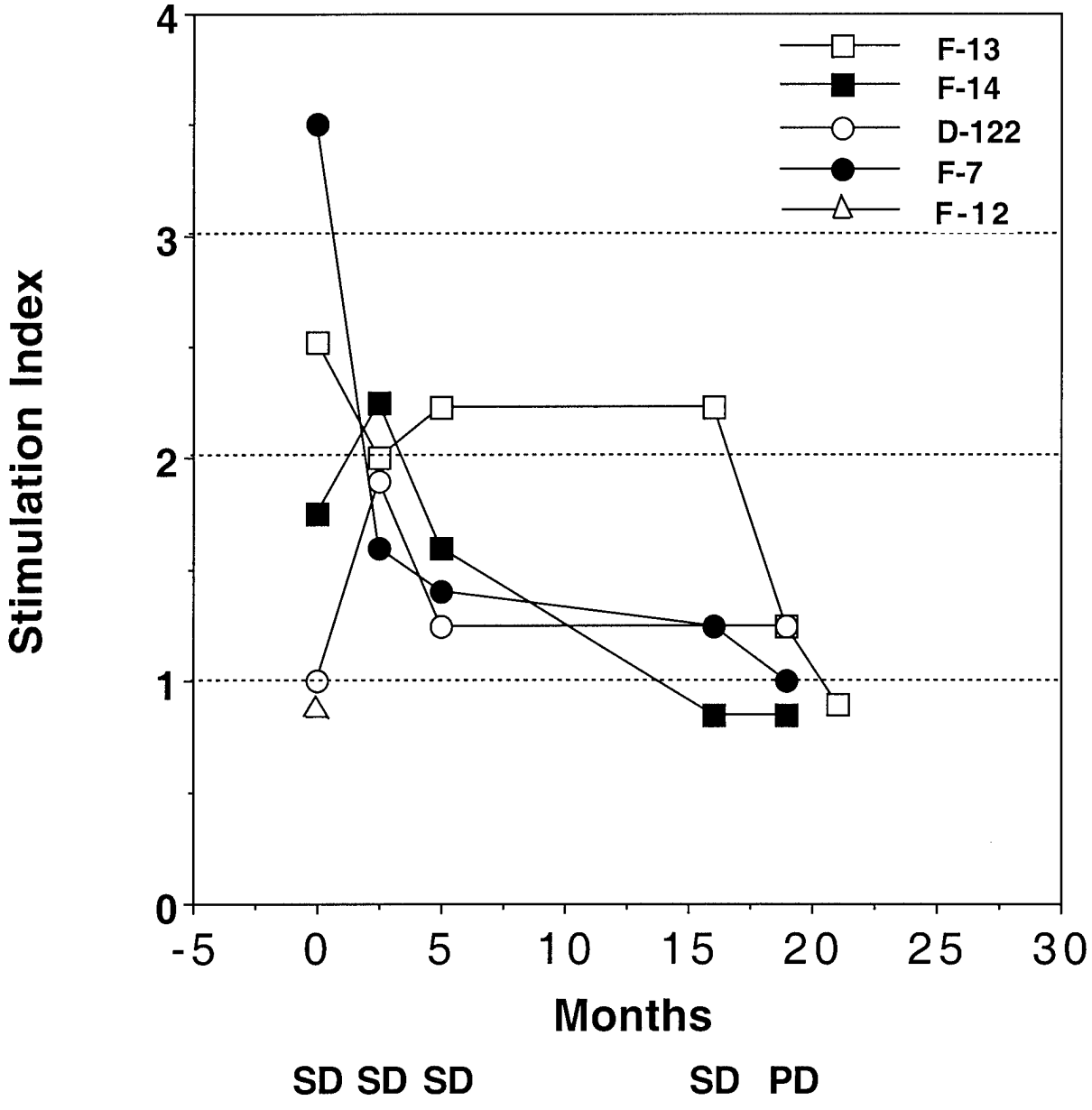
19. Tian, J., Atkinson, M.A., Clare-Salzler, M., Herschenfeld, A., Forsthuber, T., Lehmann, P.V. and Kaufman, D.L. Nasal administration of glutamate decarboxylase (GAD65) peptides induces Th2 responses and prevents murine insulin-dependent diabetes. J. Exp. Med., 183:1561-1567, 1996.

20. Topalian, S.L., Gonzales, M.J., Parkhurst, M., Li, Y.F., Southwood, S., Sette, A., Rosenberg, S.A. and Robbins, P.F. Melanoma-specific CD4+ T cells recognize nonmutated HLA-DR-restricted Tyrosinase epitopes. J. Exp. Med., 183:1965-1971, 1996.

21. Yoshino, I., Goedegebuure, P.S., Peoples, G.E., Lee, K.-Y. and Eberlein, T.J. Human tumor infiltrating CD4+ T cells react to B cell lines expressing heat shock protein 70. J. Immunol. 153:4149-4158, 1994.

22. Takahashi, T., Chapman, P.B., Yang, S.Y., Hara, I., Vijayasandhi, S. and Houghton, A.N. Reactivity of autologous CD4+ T lymphocytes against human melanoma. J. Immunol., 154:772-779, 1995.

23. Nishimura, M.I., Avichezer, D., Custer, M.C., Lee, C.S., Chen, C., Parkhurst, M.R., Diamond, R.A., Robbins, P.F., Schwartzentruber, D.J. and Rosenberg, S.A. MHC class I-restricted recognition of a melanoma antigen by a human CD4+ tumor infiltrating lymphocyte. Cancer Res., 59:6230-6238, 1999.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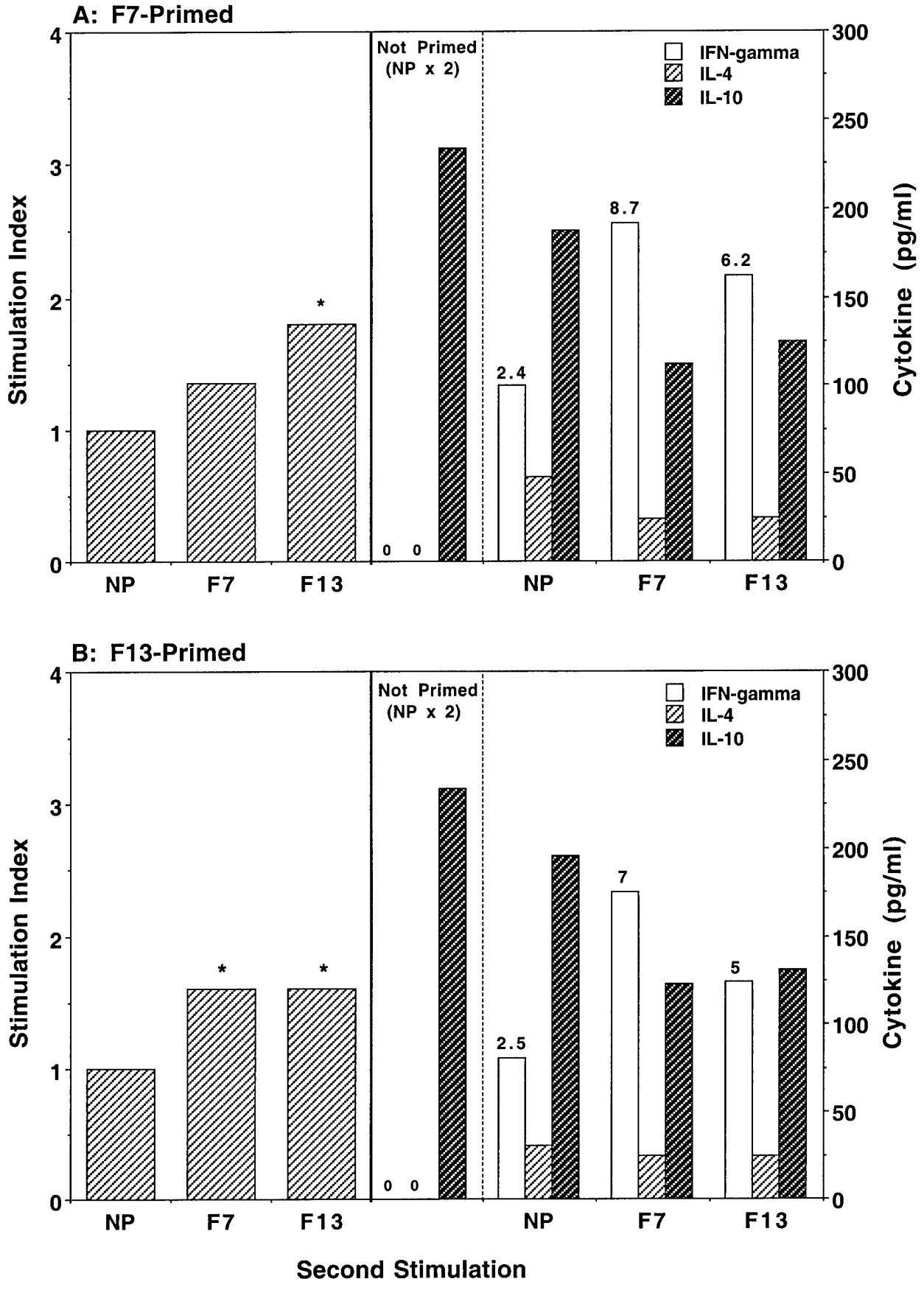
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(NP x 2)
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IL-4
IL-10
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2
3
4
50
100
150
200
250
300
*
2.4
8.7
6.2
0 0
NP
F7
F13
B: F13-Primed
Not Primed
(NP x 2)
IFN-gamma
IL-4
IL-10
Stimulation Index
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*
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7
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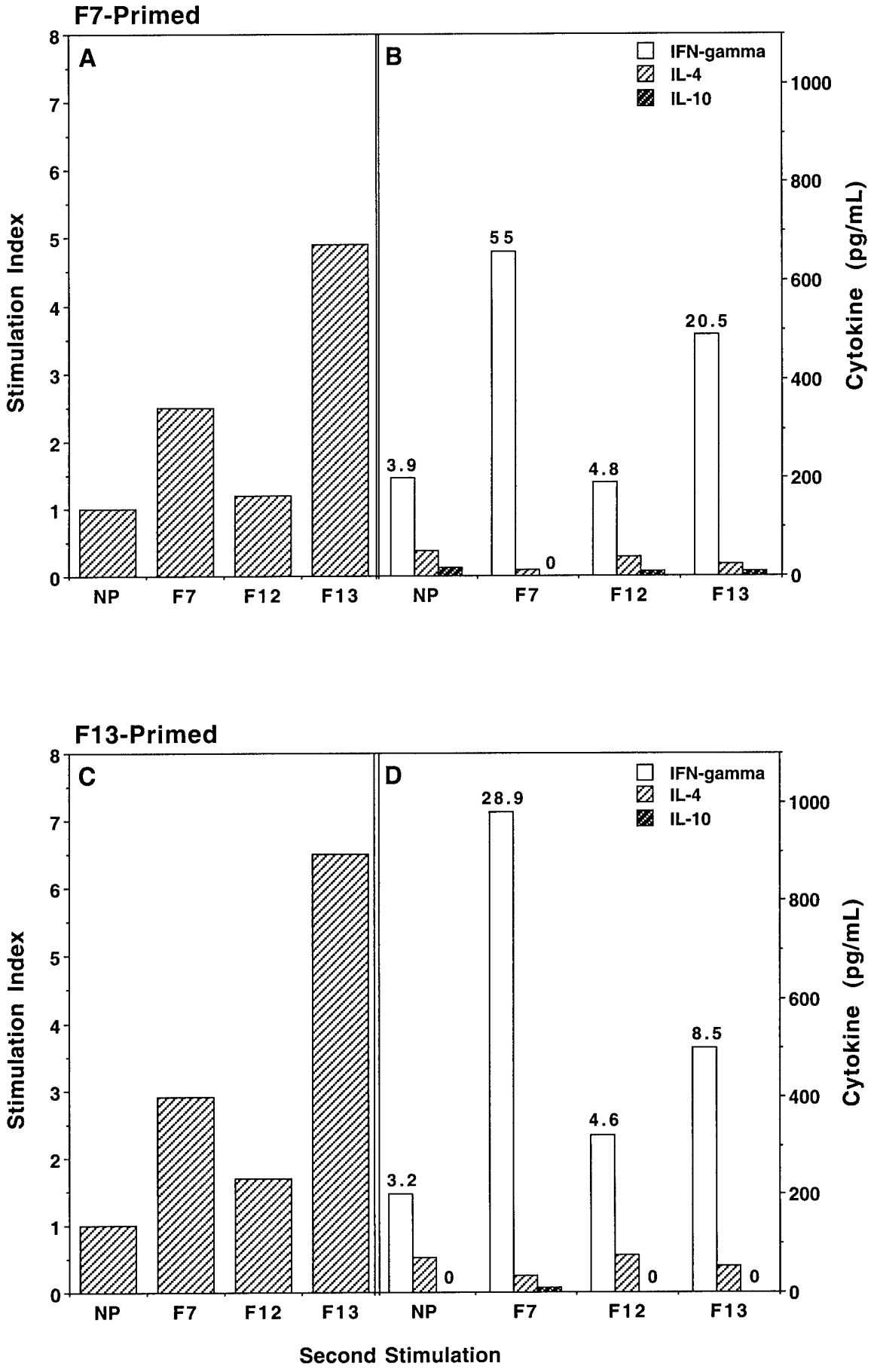
F7
F13
Second Stimulation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A
B
IFN-gamma
IL-4
IL-10
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600
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1000
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20.5
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F7
F12
F13
NP
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F12
F13
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C
D
IFN-gamma
IL-4
IL-10
Stimulation Index
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F7
F12
F13
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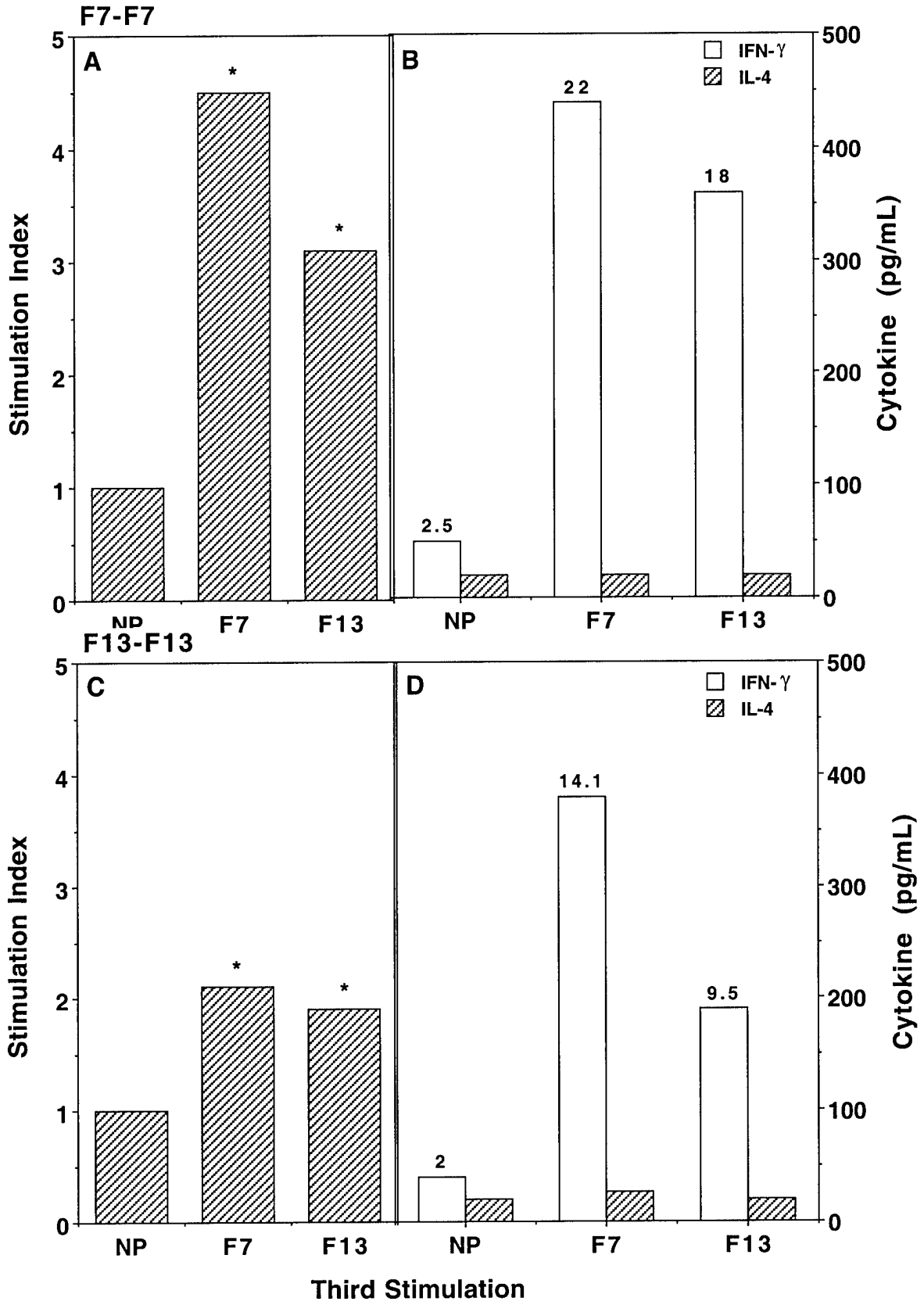
F7
F12
F13
Second Stimulation


F7-F7
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B
IFN-γ
IL-4
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F13-F13
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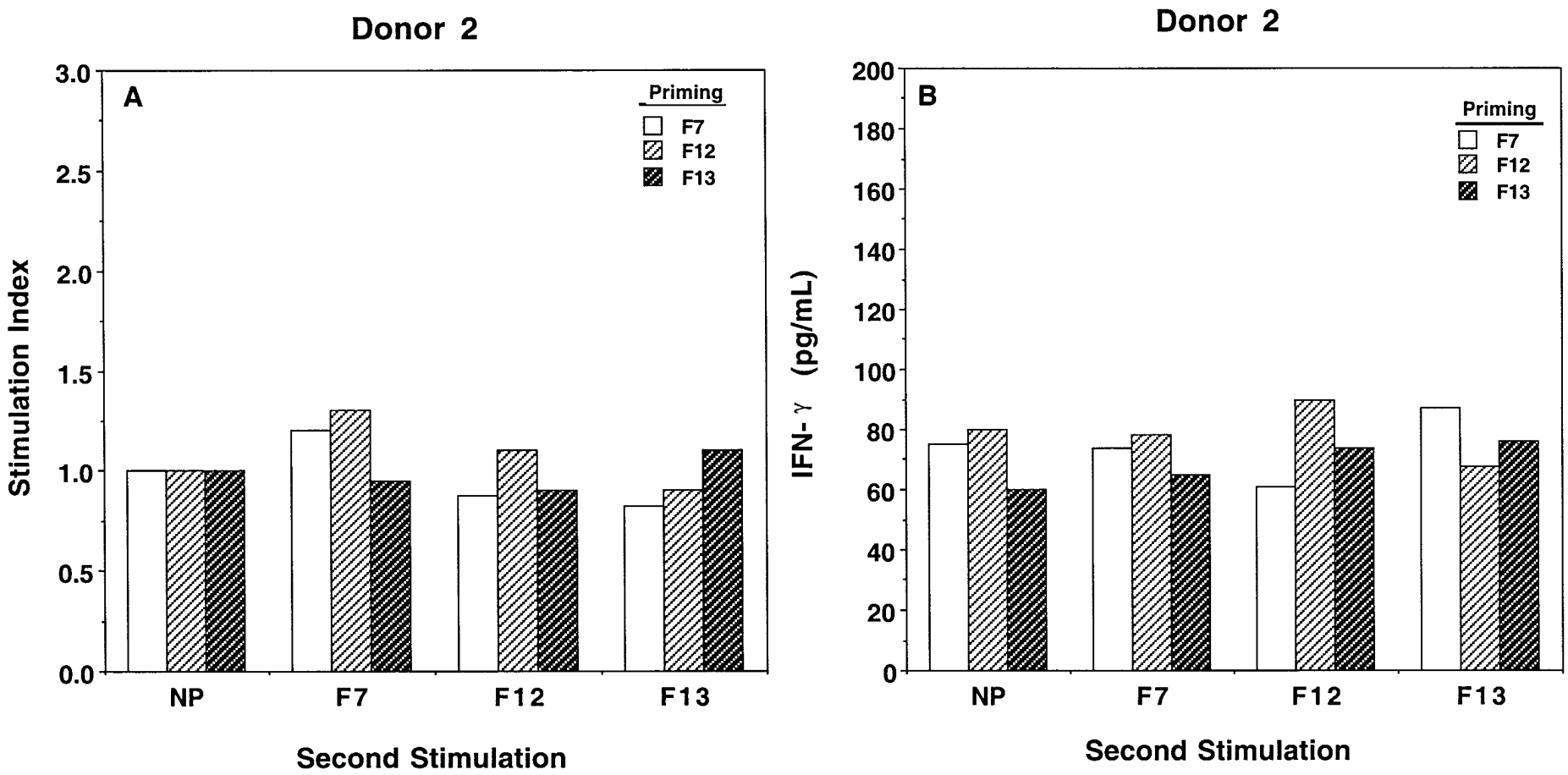
D
14.1
9.5
2
Third Stimulation


Donor 2
A
Priming
F7
F12
F13
Stimulation Index
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1.5
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0.0
NP
F7
F12
F13
Second Stimulation
Donor 2
B
Priming
F7
F12
F13
IFN-γ (pg/mL)
200
180
160
140
120
100
80
60
40
20
0
NP
F7
F12
F13
Second Stimulation

From: Henry Kuerer on 08/28/2000 06:20 PM

To: James Murray/MDACC@MDACC, Kelly K. Hunt/MDACC@MDACC, Aysegul Sahin/MDACC@MDACC

cc: Constantin G. Ioannides/MDACC@MDACC

Subject: SSO LN Abstract

Please find attached an abstract for the Society of Surgical Oncology. We would greatly appreciate any comments or suggestions by Wed am Aug 30th.

AXILLARY LYMPH NODE (LN) CELLULAR IMMUNE RESPONSE TO HER-2/NEU PEPTIDES IN PATIENTS WITH CARCINOMA OF THE BREAST

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The HER-2 peptide G89 (777-789) was recently shown to induce a proliferative response by peripheral CD4+ T cells in breast cancer patients. To investigate potential differences in the local cellular immune response in breast cancer patients with (Mets+) and without (Mets -) axillary LN metastases, lymphocytes were isolated from axillary LNs from patients with breast cancer and proliferative and cytokine response to G89 were determined.

METHODS: Freshly isolated lymphocytes from LNs of seven women with invasive breast cancer were plated at 2.5 x 105 cells/well in triplicate. Four women were treated preoperatively with adriamycin-based chemotherapy. Cells were stimulated with 15 uM G89 and G90 (886-898, control HER-2 peptide) and other control antigens. [3H] thymidine incorporation was determined 4 days later. Cytokines (Ifn-, IL-4) levels were determined at priming or at restimulation with G89 and G90 using cytokine specific double sandwitch-ELISA kits (BioSource Int, CA) with a sensitivity of 10 pg/ml. Differences between groups were compared using the Student t-test.

RESULTS: Three of six Mets(+) LNs showed a 3-4 fold higher proliferative response to G89 compared with unstimulated cells from the same LN ($P < 0.05$). Three of four Mets(-) LNs also responded to G89 indicating the presence of prior G89 sensitized and non-tolerized T cells. In one patient where there was no proliferation with G89, the ratio of Ifn- to IL-4 levels in response to G89 was > 5 compared with G90 which yielded a ratio of < 0.5. In two patients where Mets(+) and Mets(-) LNs were analyzed in parallel, the Ifn- to IL-4 ratio was 3-4 fold lower in Mets(+) LNs compared with Mets(-) LNs. Mets(+) LNs from two of the four patients who had preoperative chemotherapy responded to G89 by proliferation.

CONCLUSIONS: These results indicate that HER-2 peptide G89 can activate LN T cells from women with invasive breast cancer. This activation is associated with a predominately TH1 cytokine response and therefore suggests that conditioning with G89 may be of value in breast cancer vaccine

development.

Identification of activated tumor antigen-reactive CD8+ cells in healthy individuals

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Abstract. We investigated the ability of HER-2 peptide E75, which maps an immunodominant CTL epitope for ovarian and breast tumor-associated lymphocytes (TAL), to activate effector functions in freshly isolated CD8+ cells from healthy individuals. IFN-γ was rapidly induced by E75 within 20-24 h, in five of six healthy donors, in the presence of IL-12 and was detectable as early as 6 h. The IFN-γ levels were Ag-concentration dependent. Similar results were obtained with peptides mapping CTL epitopes from two other tumor Ag: folate binding protein (FBP) and amino-enhancer of split of Notch (AES). IFN-γ was also detected, from freshly isolated, unstimulated PBMC in response to HLA-A2 matched tumors + IL-12 but not of IL-12 alone. The major source of IFN-γ were CD45RO+ CD8+ cells. Induction of IFN-γ and IL-2 from CD8+ cells and of IL-12 from dendritic cells (DC) by CD8+ cells reactive with E75 mirrored their induction by the influenza matrix peptide (M1: 58-66) in the same individual. Responses to M1 are used to define the presence of activated memory cells in healthy individuals. Compared to M1 responses E75 recognition induced 2-4-fold lower levels of IL-12 from the same APC and IFN-γ and IL-2 from the same CD8+ cells. At lower Ag concentrations the endogenous IL-12 induced by E75-reactive CD8+ cells did not reach the threshold required to co-stimulate for IFN-γ. αB7.1 synergized with E75 in increasing the overall levels of IL-2 induced within 24 h. The presence of tumor Ag-reactive activated CD8+ cells in healthy individuals may improve our understanding of the mechanisms of immunosurveillance and regulation of immune responses by tumors.

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Abbreviations: TAL, tumor associated lymphocytes; α, anti; DC, dendritic cells; HER-2, HER-2/neu proto-oncogene; HS, human serum; NP, no peptide; AES, aminoenhancer of split; FBP, folate binding protein

Key words: CTL, HER-2, interferon-γ, IL-12, IL-2, CTLA-4, B7

Introduction

The recent characterization of tumor Ag recognized by CTL opened the possibility of development of Ag and epitope-specific cancer vaccines. Tumor Ag recognized by melanoma, ovarian, and breast CTL have been demonstrated to be self-proteins (1). The fact that in cancer patients, CTL-specific for these self peptides co-exist with progressive tumors, suggest that such responses can be primed *in vivo*, but either CTL do not expand to the numbers required to mediate an effective response, or they expand but they are not functional in their state *in vivo*. This raise the question whether tumor occurrence induced an inefficient CTL response, or subsequent to tumor progression, tumor or host derived factors suppressed a protective pre-existent CTL response, established during the life of the patient. An alternative possibility is that tumor progression coincided with exhaustion of the protective CTL response.

The information regarding the presence and functionality of activated effector and memory tumor Ag reactive CD8+ cells in healthy individuals is limited. The fact that detection of *ex vivo* activated cytolytic effctors require repeated stimulation with Ag, mitogens and cytokines, did not allow to assess the functional phenotype of these cells (2,3) However, in one extensive study, specific cytolysis against MART-1 (27-35) was detected significantly earlier in melanoma patients than in health donors indicating a lower level of sensitization of healthy individuals by tumor Ag than of cancer patients (4). This also suggested that either different tolerance mechanisms are operative in maintaining the unresponsive state in healthy donors compared with cancer patients or these cells, in healthy individuals, are hyporesponsive to Ag in terms of cytolytic activity and possibly proliferation compared with cancer patients.

The possibility that these cells are hyporesponsive to Ag suggests that some tumor Ag may be partial or weak agonists for activation of a specific functions. If this is the case, in the presence of high Ag levels originated from tumor cells, these cells may expand, while in healthy donors the low endogenous Ag levels may be unable to expand these cells. However, the low Ag levels may activate effector functions different from cytolysis. Since vaccination strategies aim to induce long lived memory against tumors, the presence of activated effectors in healthy donors may be significant for understanding tumor immunosurveillance and its relevance to protective tumor immunity (5).

To address the presence of activated effectors in the absence of disease, and establish the ability of tumor Ag to activate the effector functions of $CD8^+$ cells, we investigated the ability of peptide E75, HER-2 (369-377) (6), to stimulate cytokine induction within less than 24 h in freshly isolated, peripheral blood $CD8^+$ cells from healthy individuals, when pulsed on $CD13^+$ $CD14^-$ DC (DC-E75). E75 is not only recognized by *in vivo* generated ovarian CTL-TAL, but *in vitro* E75 restimulated, *ex vivo* primed T-cells from cancer patients mediate specific tumor lysis (7,8). We used as antigen presenting cells (APC) autologous $CD14^+$ derived dendritic cells (DC) because of their reported higher presenting ability than other APC.

Since the frequency of $CD8^+$ cells bearing TCR capable of recognizing tumor Ag may be low and primary stimulation of PBMC with most tumor Ag, including HER-2 is inefficient in induction of detectable specific cytolytic effectors we focused our analysis on cytokine induction by peptides corresponding to CTL epitopes from HER-2 (6-9) and the newly identified tumor Ag: folate-binding protein (FBP) and amino-enhancer of split (AES) in PBMC from healthy donors (10,11). This analysis can detect response patterns to Ag, the presence of activated memory effectors (12), the ability of the tumor peptide to induce a type 1 (inflammatory response), and the requirements for co-stimulation for amplification of this response. The advantage of this model system is that the effects of defined tumor Ag on activation of peripheral T-cells from healthy donors are not perturbed, or polarized by prior *in vitro* culture with Ag + cytokines. This also adds a component of physiologic relevance to the activation pathways investigated since Ag + cytokine induced activation may be critical to the patient's response to tumors.

We rationalized that the patterns of response to Ag should be indicative of the nature of responders in the population as follows: If the Ag targets naive cells, they will respond if costimulatory receptors are present on APC and bind to their appropriate ligands. An effector response by cytokines will be observed after progression through the cell cycle and 2-4 divisions (i.e., 30-40 h) (13). This response will be inhibited by antibodies to costimulatory molecules such as B7.1/B7.2. If Ag induces tolerance, then naive cells may express a partial response at priming but they will not develop a response at restimulation (14). If activated effectors are present and are tolerized/anergized by exposure to Ag in the absence of costimulation, they will be unable to respond to the cognate/crossreactive stimulus that was initially effective for their activation. Analysis of the cytokine response can distinguish whether Ag induces anergy (characterized by minimal IL-2 secretion) or cytokine mediated immunosuppression due to high levels of IL-10 (12-14). In contrast, if activated effectors are present, they will immediately or rapidly respond to Ag by cytokine secretion without requiring division (15). In this case costimulation through surface receptors may have an enhancing/stabilizing effect on some responses (e.g., CD28 on IL-12R expression) and a regulatory effect on other responses (e.g., proliferation) due to B7 ligation by negative signaling receptors (e.g., CTLA-4) present on activated cells (16). Thus characterization of the patterns of reactivity of PBMC to tumor Ag can provide an answer to these questions.

DC-E75 stimulated high and rapid Ag specific IFN-γ, secretion by these $CD8^+$ cells in most donors in the presence of IL-12. DC-E75 also induced IL-2 in these cells while αB7.1 enhanced IL-2 production. The tumor Ag reactive cells were below the affinity threshold for triggering IL-12 production by DC. Similarly the inhibition of IL-2 induction by B7-CTLA-4 interaction helps maintaining these cells in a functionally competent but low reactive state. The primary DC-E75 stimulation even in the presence of αB7.1, or α-CTLA-4 enhanced weakly T-cell proliferation and did not enhance specific cytotoxicity. Our results indicate that activated effector cells reactive with E75 and other tumor Ag are frequently present in healthy donors. These cells appear to be neither Ag ignorant nor functionally anergized, but the tumor Ag acts as a weak/partial agonist by selectively inducing only a subset of $CD8^+$ effector functions.

Materials and methods

Cells, antibodies and cytokines. $HLA\text{-}A2^+$ PBMC were obtained from healthy volunteers from the Blood Bank of M.D. Anderson Cancer Center. The HLA phenotypes of the donors used in this study are as follows: donor 1 (A2, B7, 44), donor 2 (A2, 33, B40, 44), donor 3 (A2, 33, B41, 81), donor 4 (A1, 2, B27, 44), donor 5 (A1, 2 B44, 57, Cw5, 6), donor 6 (A2, 31, B35, 44, Cw4, w5). T2-cells, ovarian SKOV3, SKOV3.A2 cells, and indicator tumors from ovarian ascites were described (9). mAb to CD3, CD4, CD8 (Ortho), CD13 and CD14 (Caltag Laboratories, San Francisco, CA), B7.1 and B7.2 (CD80 and CD86, Calbiochem), ICAM-1 (ICAM-1 (CD54, Calbiochem), CD40L (Ancell, Bayport, MN), HLA-A2 (clone BB7.2, ATCC), and MHC-II (L243, Dako Corp., Carpintera, CA) were used as unconjugated FITC or PE conjugated. Anti-CTLA-4 was a kind gift from Dr Peter Linsley (Bristol-Myers, Seattle, WA). mAb specific for IL-12, IFN-γ and isotype controls were obtained from Pharmingen. The following cytokines were used: GM-CSF (Immunex Corp., Washington, DC), specific activity 1.25×10^7 CFU/250 mg; TNF-α (Cetus Corp., Emeryville, CA), specific and activity 2.25×10^7 U/mg, IL-4 (Biosource International), specific activity, 2×10^6 U/mg. IL-2 (Cetus Corporation) specific activity 18×10^6 IU/mg, IL-12 of specific activity 5×10^6 U/mg was a kind gift from Dr Stanley Wolf, Department of Immunology, Genetics Institute, Cambridge, MA.

Synthetic peptides. The HER-2 peptides used were: E75 (369-377) GP2/F53: (IISAVVGIL, 654-662), and F57 (IHLNGSAYSL, 439-447). GP2 and F57 define HER-2 CTL

epitopes distinct from E75 (8-9). The modified Muc-1 peptides used were D125: (GVTSAKDTRV) and D132 (SLADPAHGV). The FBP peptides used were: E39 (FBP,191-199, EIWTHSYKV), and E41 (FBP, 245-253 LLSLALML). The Amino Enhancer of Split (AES) peptide used was G76:GPLTPLPV. FBP and AES peptides were recently identified to be recognized by ovarian and breast CTL (10,11). The positive control CTL epitope used was the influenza matrix peptide (58-66): GILGFVFTL, designated as M1. M1 forms an immunodominant epitope recognized by memory CTL in healthy donors (15). All peptides were prepared by the Synthetic Antigen Laboratory of M.D. Anderson Cancer Center and purified by HPLC. Peptides were 95-97% pure by amino acid analysis. Peptides were dissolved in PBS and stored frozen at -20°C in aliquots of 2 mg/ml.

Immunofluorescence. Antigen expression by DC and T-cells was determined by FACS using a flow-cytometer (EPICS - Profile Analyzer, Coulter Co., Hialeh, FL). DC were defined by the presence of CD13 and absence of CD14 marker after culture in GM-CSF and IL-4. For phenotype analysis, DC were incubated with PE-conjugated anti CD13 mAb and FITC-conjugated mAb specific for a surface Ag. For determination of the effects of cytokines, peptides and T-cells on surface antigen expression, DC were incubated with the same amounts of cytokines and peptides as in T-cell activation assays for 24 h, and the levels of Ag expression were determined in the gated $CD13^+$ population.

Culture of PBMC-derived DC. $CD13^+$ DC were generated from freshly isolated PBMC following the established CD14 methods (17,18). Complete RPMI medium (containing 10% FCS) supplemented with 1,000 U/ml GM-CSF or 500 U/ml IL-4 was added to each well containing plastic-adherent cells and maintained for 7 days. T-cells were obtained from the plastic non-adherent PBMC by removal of $CD16^+$ and $CD56^+$ cells. $CD8^+$ cells were isolated by removing first the $CD4^+$, and then the $CD16^+$ and $CD56^+$ cells from the non-adherent population using Dynabeads (Dynal, Oslo, Norway). $CD8^+$ subpopulations were obtained using anti-CD45RO mAb and anti-CD45RA mAb (UCHL-1, Dako) as described (15). After depletion, the resulting cells were 97% $CD8^+$ as determined by flow cytometry.

T-cell stimulation by peptide pulsed DC. DC were plated at $1.2x10^5$ cell/well in 24-well culture plates, and pulsed with peptides at 50 μg/ml in serum-free medium for 4 h before addition of responders. TNF-α (50 U/ml) was added to DC for the last hour to stimulate Ag uptake and presentation (17). Autologous, isolated $CD8^+$ cells or isolated $CD8^+$ cells ($CD45RO^+$ and CD45RO + cells depleted) in RPMI 1640 containing 10% HS were added to DC at $1.5x10^6$/ml, followed by IL-12. IL-2 was added 12-16 h later to each well. For inhibition studies, mAb specific for B7.1, B7.2, HLA-A2 and isotype control MOPC myeloma were added to DC or tumor cells, 1 h before responders in amounts reported to be inhibitory by the manufacturers. Anti CTLA-4 and CD40L mAb were added to T-cells 1 h before they were added to cultures. The effects of peptides and cytokines on T-cell survival were determined by counting the numbers of recovered viable cells, and determining the numbers of $CD8^+$ and $CD4^+$ cells in the sample by flow cytometry. Specific proliferative responses to E75 were determined by measuring the incorporated radioactivity in equal cell numbers pulsed with 1 μCi of (^{3}H)-TdR (19).

CTL and cytokine assays. Recognition of peptides used as immunogens by CTL was performed as described (8). Equal numbers of viable effectors from each well were used in all assays. Supernatants collected at 6, 24, or 48 h were tested in duplicate for the presence of IL-2, IL-4, IL-10, IL-12 and IFN-γ using cytokine ELISA-kits (Biosource International, Camarillo, CA) or R&D systems as described (19) with a sensitivity of 4-7 pg/ml. IL-12 was detected using an ELISA kit which recognizes both p40 and the natural heterodimeric molecule.

Results

$CD8^+$ cells from healthy donors display specific IFN-γ secretion within 24 h of contact with HER-2 peptide E75 potentiation by IL-12. To address whether stimulation with E75 induce cytokine responses, plastic-non-adherent PBMC from healthy donors were stimulated with autologous DC pulsed with E75 (DC-E75) or as control with DC which were not pulsed with peptides (DC-NP). Supernatants were collected 20-24 h later and tested for the presence of IFN-γ. Since the frequency of E75-specific responders may be low, we rationalized that addition of low concentrations (100-300 pg/ml) of IL-12 will amplify the levels of IFN-γ induced by E75, thus increasing the likelihood of detection of E75-responsive T-cells. IL-12 acts as co-stimulator for IFN-γ induction from T-cells by Ag, but by itself is a weak inducer of IFN-γ in T-cells (20,21). Results in Fig. 1 show the pattern of IFN-γ responses to E75 from four healthy donors, in the absence or presence of IL-12. In donor 1 in some experiments performed over time E75 rapidly induced IFN-γ without exogenous IL-12 (Fig. 1A), while in donor 2 (Fig. 1B) IL-12 was required to induce detectable IFN-γ levels to E75.

Since these experiments were performed with populations and not with isolated $CD8^+$ cells, these experiments were repeated three times at weekly intervals with donor 3 to address whether rapid IFN-γ induction and its amplification by IL-12 was not an isolated event. From donor 3 it was possible to obtain repeatedly peripheral blood over six months. Most experiments shown here were performed with donor 3 and were confirmed with at least one randomly selected $HLA-A2^+$ donor. Each experiment shown in Fig. 1C was performed with blood samples collected in different days. We used each time freshly cultured DC, plastic non-adherent PBMC from the same sample, and since the amount of blood was small, variable concentrations of E75 (20-50 μg/ml) and IL-12 (150 or 300 pg/ml). The results of stimulation experiments over a period of 3 weeks confirm that the pattern of responses observed with donors 1 and 2 was not an isolated event. At these E75 concentrations, IFN-γ was undetectable unless its induction was amplified by IL-12. In donor 5 E75 in high concentrations (100 μg/ml) induced high levels of IFN-γ within 24 h in the absence of exogenous IL-12

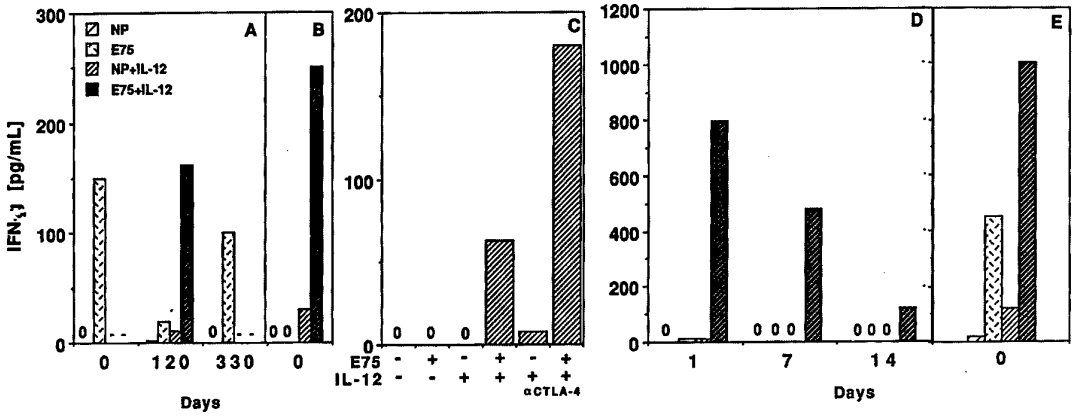

Figure 1. Freshly isolated, unstimulated plastic non-adherent PBMC from healthy donors (A, B, D and E) and breast cancer patients (C) specifically secrete IFN-γ within 20 h in response to E75. A, Donor 1. PBMC were collected on days 0, 120, and 330 counting as day 0 the date of first collection and stimulated with E75 at 50 μg/ml. (-) indicate not tested. B, Donor 2. E75 at 50 μg/ml. C, Unimmunized breast cancer patient. Supernatants were collected at 24 h after stimulation with 50 μg/ml E75. IL-12 was used at 3 U (330 pg/ml). D, Donor 3. Days 7 and 14 indicate the days after the first stimulation when the experiment was repeated with fresh PBMC. Day 1, 50 μg/ml E75 + 330 pg/ml IL-12; day 7, 25 μg/ml E75 + 150 pg/ml IL-12; day 14: 20 μg/ml E75 + 150 pg/ml IL-12. E, Donor 5, 100 μg/ml E75 + 330 pg/ml IL-12. Differences between the levels of IFN-γ induced by E75 + IL-12 and E75 alone or IL-12 alone were considered significant ($p<0.05$).

(Fig. 1C, column E). Similar results were obtained with donor 4 (Fig. 6A) which was tested in a separate experiment but not with donor 6 (Fig. 3B).

IL-4 was not detected in the E75 stimulation supernatants while the levels of IL-10 determined in the same experiment did not exceed 10 pg/ml during the first 96 h of E75-stimulation (data not shown). Similar results were obtained with 5 of 6 unimmmunized HLA-A2$^+$ breast cancer patients tested in the same conditions. Representative results with one patient in Fig. 1C also show that the IFN-γ response was also enhanced by α-CTLA4 mAb. These results suggest that there are significant numbers of E75-reactive T-cells in the PBMC of unimmunized healthy individuals and cancer patients that could be readily recalled following a primary stimulation *in vitro* by E75 ± IL-12.

T-cells from healthy donors secrete IFN-γ within 24 h in response to tumor cells in the presence of IL-12. Identification of E75 reactivity with high frequency in the PBMC raise the question of the potential of these cells for tumor recognition. To address this question, we investigated whether freshly isolated donor 1 T-cells recognized better the HLA-A2 matched tumor SKOV3.A2 compared with the non-matched SKOV3. SKOV3 and SKOV3.A2 are identical but the latter express a transfected HLA-A2 gene. Thus, donor 1 and SKOV3.A2 (HLA-A2, 3, 28, B18, 35) shared only HLA-A.2. We wanted to know whether the same or higher levels of IFN-γ will be induced in T-cells responding to Ag presented by allo-MHC-I compared with common HLA-A2. The results, Fig. 2A show that T-cells secreted low IFN-γ levels in response to SKOV3.A2. IFN-γ was not detected in response to SKOV3. In the presence of IL-12, IFN-γ was detected in both cultures within 24 h, but the levels were significantly higher in response to SKOV3.A2 than to SKOV3, suggesting that HLA-A2 restricted activated T-cells are present in this donor. To verify that IFN-γ production was the result of

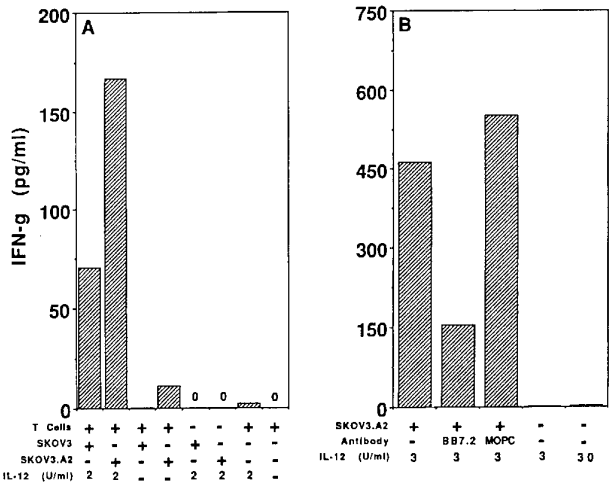

Figure 2. A, Freshly isolated T-cells from donor 1 produce higher levels of IFN-γ within 24 h of co-culture with SKOV3.A2 compared with SKOV3. ($p<0.05$, by the Student's t-test). B, IFN-γ response to SKOV3.A2 is dependent on HLA-A2 recognition. Experimental conditions as described in the Materials and methods.

HLA-A2 recognition, SKOV3.A2 cells were incubated either with BB7.2 mAb (α-HLA-A2) or with a non-specific isotype control Ab (MOPC). The results (Fig. 2B) confirmed that most of the IFN-γ was produced in response to HLA-A2, and was not increased by higher concentrations of IL-12. Thus, freshly isolated T-cells from healthy donors can recognize tumors in an MHC restricted fashion without previous *in vitro* stimulation, suggesting that activated effectors are present in these cells.

E75 induce IFN-γ secretion from activated memory cells. Requirement for cognate Ag. Rapid induction of IFN-γ by

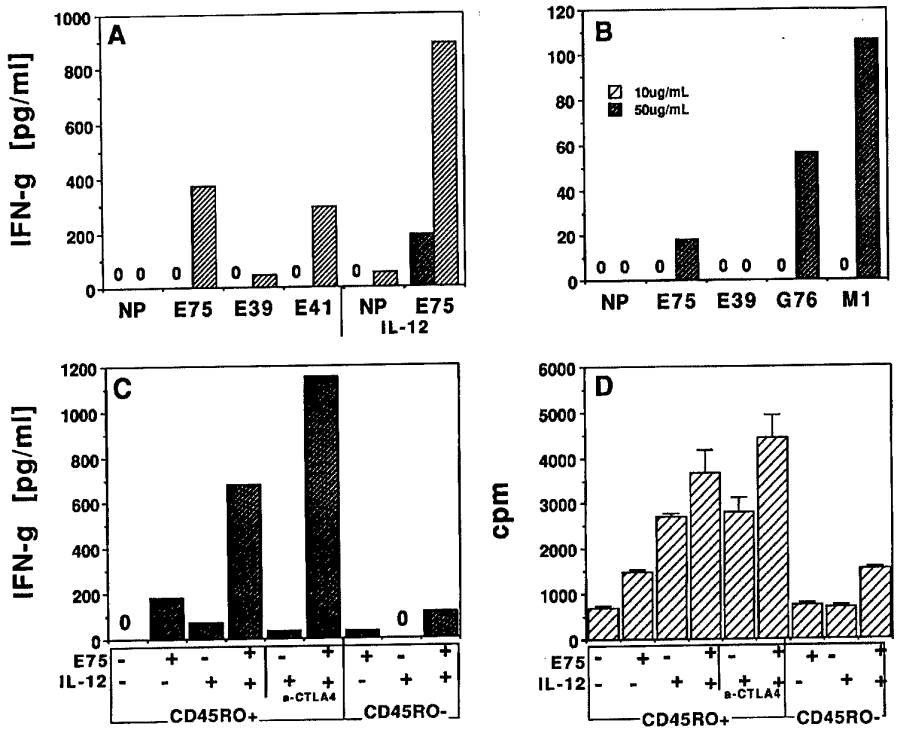

Figure 3. A, Induction of IFN-γ by E75 within 6 h (narrow hatching), and 48 h (wide hatching) from isolated CD8+ cells from donor 3. All peptides were used at 10 μg/ml; IL-12 at 330 pg/ml. B, IFN-γ induction from donor 6 by tumor peptides E75, E39, G76 and positive control M1 within 20 h in the absence of exogenous IL-12 is Ag and its concentration dependent. IFN-γ by G76 and M1 was significantly different from control NP cultures ($p<0.05$). C, IL-12 and αCTLA-4 mAb synergize in enhancing IFN-γ induction in response to E75 in isolated CD8+ CD45RO+ cells from donor 3. Equal numbers (10^6) of CD8+ CD45RO+ and CD8+ CD45RO cells were used as responders in each well. D, E75 in the presence of IL-12 and αCTLA-4 enhanced only marginally CD8+ cells proliferation. Equal numbers of CD8+ CD45RO+ and CD45RO+ depleted (CD45RO-) were collected from cultures after 48 h and used to determine differences in the rate of proliferation by E75. 10^5 donor 3 live cells were incubated with of ^{3}H-TdR for 8 h. Differences between IFN-γ levels in columns 2, 3, 4, 5, and 6 are significant ($p<0.05$). The experiment was performed in tetraplicate. Differences in ^{3}H-TdR are significant for all the (±) E75 groups by the Student's t-test, but the stimulation indexes (SI) are ≤2.0. Differences between groups IL-12-αCTLA4, and IL-12 alone are not significant, similarly differences between E75 + IL-12 and E75 + IL-12 + αCTLA-4 are not significant.

E75 raised the questions whether E75 and other tumor peptides prime naive T-cells or whether activated T-cells of this specificity are present in these healthy donors. Naive T-cells and resting memory cells require cell cycling (at least 1-2 divisions) i.e., minimum 30-40 h after Ag stimulation to secrete IFN-γ, while activated memory effector CTL respond to Ag by IFN-γ without requirements for additional cycling (i.e., O divisions) (13). Thus from memory effectors IFN-γ can be detected within 6-24 h (15).

To establish whether the rapid IFN-γ induction by E75 is a property of existent activated CD8+ cells and not the result, of initiation of activation of naive cells, isolated CD8+ cells from donor 3 were stimulated with DC-E75 in the presence or absence of IL-12. Supernatants were collected 6 h later and analyzed for IFN-γ. Rapid induction of IFN-γ within 6 h was observed only in cultures containing E75 + IL-12 (Fig. 3A). The levels of IFN-γ continued to increase over the next 48 h. At this time, IFN-γ was detectable even from the cultures that did not receive IL-12. The levels of IFN-γ were dependent on Ag sequence, since FBP peptides, E39 and E41, mapping CTL epitopes showed similar (E41) or weaker (E39) abilities than E75 to induce IFN-γ. Secretion of IFN-γ was Ag concentration dependent, indicating that E75 specific T-cells and not NK cells were the source of this cytokine (presented from a separate experiment in Fig. 5). Similar rapid IFN-γ induction by E75 + IL-12 within 12 h was observed with donors 1 and 4 (not shown) confirming that activated E75-reactive T-cells are present in healthy individuals.

To verify that the IFN-γ induction in tumor Ag-reactive CD8+ cells from PBMC is as rapid as the response of activated T-cells reactive with conventional Ag, the experiment was repeated in the absence of IL-12 with CD8+ cells from donor 6, using E75, E39, and the AES peptide G76 as stimulators. The dominant HLA-A2 restricted CTL epitope from influenza matrix (M1:58-66) was used as positive control. A rapid IFN-γ response to M1, is commonly used to define the presence of activated memory cells to influenza in healthy individuals (15). All peptides were used at the same concentration. The results in Fig. 3B show that in this donor, G76, and M1 at 50 μg/ml (~50 μM) induced IFN-γ within 20 h even without exogenous IL-12. At 10 μg/ml none of these Ag induced detectable IFN-γ. The magnitude of response was Ag dependent. M1 induced the highest levels of IFN-γ. The IFN-γ levels induced by G76 were significantly lower. The response to E75 was borderline. These results also show that the response is peptide-specific since E39 did not induce IFN-γ.

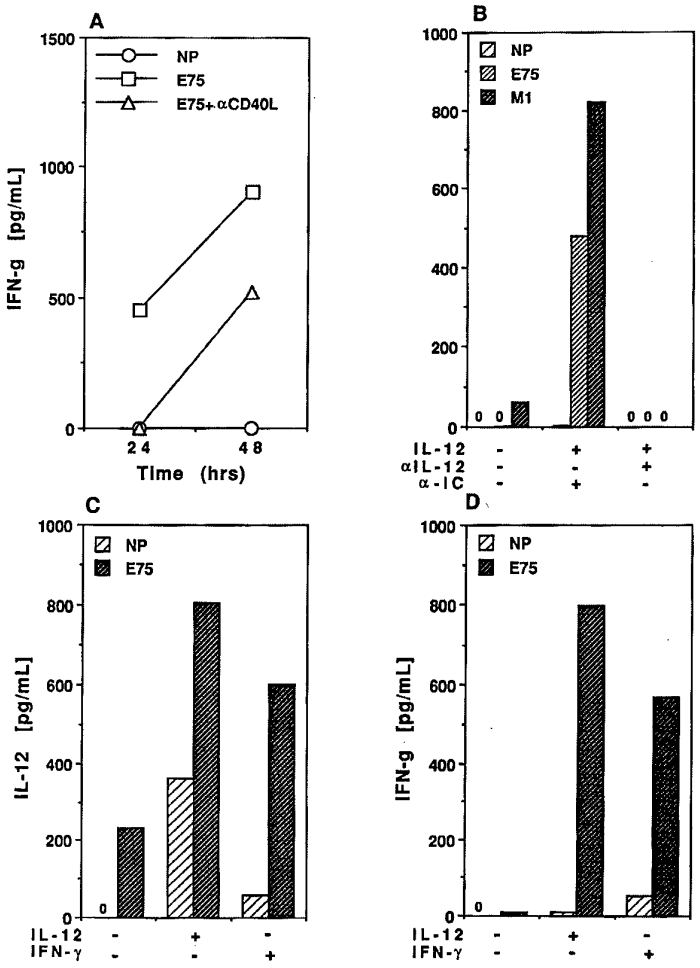

Figure 4. Induction of IFN-γ from E75-reactive CD8⁺ cells requires CD40L-CD40 interactions and is controlled by IL-12. A, Isolated CD8⁺ cells from donor 1 were stimulated with 100 μg/ml E75 in the presence of αCD40L mAb or isotype control mAb (NP) indicates background levels from cultures not stimulated with peptide (B). The same responders were stimulated with E75 and M1 at 25 μg/ml in the absence (-) or presence (+) of IL-12 (330 pg/ml). α-IC indicate isotype control Ab. The levels of IFN-γ induced by M1 were significantly higher than the levels induced by E75 in the presence or absence IL-12 ($p<0.05$). C and D, In a separate experiment responders were stimulated with E75 at 25 μg/ml in the absence (-) or presence (+) of IL-12 (330 pg/ml) or IFN-γ (50 pg/ml). NP indicate no peptide.

To establish that IFN-γ is induced in response to cognate Ag and confirm that the differences between the levels of IFN-γ are dependent on the Ag, the experiment was repeated with donor 5, using three HER-2 peptides (E75, GP2 and F57) reported to be recognized by CTL-TAL (10-12) and as control the unnatural Muc-1 peptide D132. D132 was obtained by replacing Pro (P2, P4) with a P2 anchor (L) to ensure HLA-A2 binding and a charged residue in P4 (D) to perturb a TCR contact in the corresponding Muc-1 sequence. The HLA-A2 stabilizing ability of these peptides decreased in the order F57>D132>E75>GP2. The IFN-γ levels in the presence of IL-12 were: D132:105 (unnatural peptide), F57:380, GP2:740, and E75:980 pg/ml, respectively. These results were confirmed with donor 1: NP, 20 pg; D132, 25 pg; E75, 160 pg. The rapid IFN-γ response was 6-9-fold higher for E75, previously reported to be recognized by TIL/TAL, than for the unnatural peptide D132 which is not present in the donor. Since these differences were observed at the same Ag concentration, they likely reflect differences in peptide stimulatory potency and/or frequency of existent Ag-specific activated responders.

To establish that the IFN-γ response to tumor Ag originated from effector or memory cells, but not from naive cells, CD8⁺ CD45RO⁺ and CD8⁺ CD45RO⁻ cells were isolated from the same blood sample from donor 3. Both memory and effector cells express the CD45RO antigen. Equal numbers of each population were tested in parallel for IFN-γ induction and proliferation in response to E75 ± IL-12 (Fig. 3C and D). The results show that CD8⁺ CD45RO⁺ cells were the main producers of IFN-γ in response to E75 + IL-12. The levels of IFN-γ were by 5-fold lower when CD8⁺ CD45RO⁻ cells were used as responders. Comparison of the IFN-γ levels with the proliferative response demonstrated that E75 is a weak inducer of proliferation in both CD45RO⁺ and CD45RO⁻ cells. IL-12 did not synergize with E75 in increasing CD8⁺ cells proliferation. Although the overall levels of ^{3}H-TdR incorporation were higher in the presence than in the absence of IL-12, the stimulation indexes for E75 + IL-12-stimulated

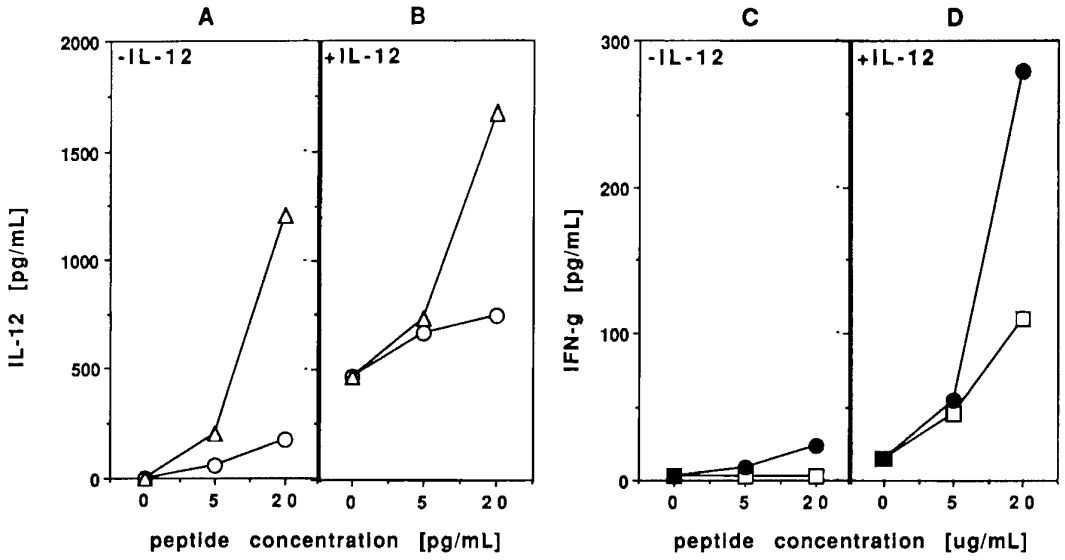

Figure 5. Concentration-dependent induction of IL-12 (A and B) and IFN-γ (C and D) by E75 and M1. ±IL-12 indicate that exogenous IL-12 was not added (-) or used (+) at 330 pg/ml. IL-12 and IFN-γ were determined from the same experiment from the same supernatants collected 20 h after stimulation. (○, ●), E75; (△, ▲), M1.

cultures were <2.0. In the presence of IL-12, blocking of CTLA-4 in responders increased the IFN-γ response to E75 compared with E75 + IL-12 by two fold. In the same experiment, the $CD8^+$ $CD45RO^+$ cells proliferation to E75 + IL-12 + αCTLA4 was not significantly different from E75 + IL-12.

E75-dependent induction of IL-12 from DC. Synergy with IFN-γ. To elucidate the IFN-γ induction pathways activated by E75 we first determined whether IFN-γ induction required CD40L-CD40 interactions between APC and T-cells. Isolated $CD8^+$ cells from donor 3 were stimulated with 100 μg/ml E75 in the absence of IL-12. Usually at this Ag concentration IFN-γ could be detected in the absence of exogenous IL-12 (Fig. 1C). αCD40L and isotype control Ab were added to cultures stimulated in parallel, and the IFN-γ levels were determined at 24 and 48 h. The results (Fig. 4A) show that IFN-γ secretion was significantly inhibited in the presence of αCD40L suggesting that IFN-γ induction required CD40L-CD40 interactions between activated T-cells and APC.

Since the CD40-CD40L interaction is the major pathway for T-cell dependent IL-12 induction from APC, this raised the question whether IFN-γ induction is controlled through IL-12 induced from APC. To address this question we determined the effects of neutralizing IL-12 on IFN-γ induction. Parallel cultures were stimulated with E75 or M1 in the presence of a neutralizing α-IL-12 mAb, and an isotype control mAb (IC). E75 and M1 were used at 25 μg/ml. At this concentration E75 but not M1 required exogenous IL-12 to detect induced IFN-γ. The results in Fig. 4B show that IFN-γ production in response to both E75 + IL-12 and M1 + IL-12 was completely inhibited by α-IL-12, suggesting that induction of IFN-γ is dependent on IL-12.

To address whether E75-reactive $CD8^+$ cells induced IL-12 from DC, we determined the levels of IL-12 in the same experiment in response to E75 and control (no peptide). In addition, we tested in parallel whether IL-12 and IFN-γ are cofactors for IL-12 induction by E75. E75 rapidly induced IL-12 (Fig. 4C). Exogenous IL-12 had a modest synergistic effect with endogenous IL-12 in determining the overall IL-12 levels in the culture (240 pg endogenous + 360 pg exogenous = 600 pg, compared with 780 pg total detected). This suggested that the co-stimulatory effect of exogenous IL-12 is not due to its own amplification. In contrast, IFN-γ at 50 pg/ml (the level induced by M1 in the absence of IL-12 in Fig. 4B) synergized with E75 in enhancing IL-12 levels: 240 + 50 = 290 pg vs. 600 pg/ml total detected (Fig. 4C, column 3). Higher levels of IFN-γ were also detected in the wells stimulated with E75 + 50 pg IFN-γ, but not in the well stimulated with IFN-γ alone, demonstrating that IFN-γ can amplify its own response only in the presence of the tumor Ag (Fig. 4D). This effect did not require exogenous IL-12 because the levels of IL-12 induced by E75 + IFN-γ (shown in Fig. 4C) were apparently above the threshold needed to costimulate IFN-γ induction.

These results suggested that E75-reactive $CD8^+$ cells induced IL-12, when recognized E75 on DC. Since IFN-γ was not detected at 25 μg E75 in this experiment, this suggested that compared with M1 the induced IL-12 levels were insufficient to co-stimulate IFN-γ. To address this question we determined in parallel the levels of IL-12 and IFN-γ induced in a concentration-dependent fashion by E75. The experiment was performed in the absence or presence of exogenous IL-12. We used M1 as a positive control. IL-12 production was Ag concentration dependent for both peptides (Fig. 5A). The levels of IL-12 induced by M1 were significantly higher than the levels induced by E75. Exogenous IL-12 did not change the dose-response pattern of IL-12 induced by the either Ag suggesting that within 24 h it did not induce higher levels of IL-12 by itself. Again M1 induced low levels of IFN-γ in the absence of exogenous IL-12.

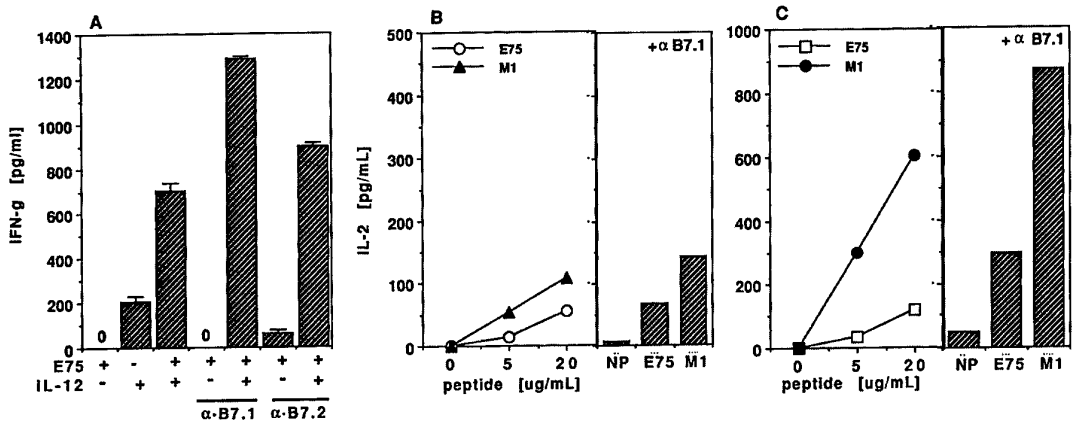

Figure 6. A, The synergy between E75 and B7.1/B7.2 mAb in enhancing IFN-γ requires the presence of IL-12. In the absence of IL-12, low levels of E75 induced IFN-γ were detected in the presence of αB7.1 at 48 h (50 pg/ml). B and C, E75 synergize with αB7.1 in enhancing IL-2 induction. Exogenous IL-2 was added at 200 pg/ml, 8 h before supernatant collection. C, Results indicate pg/ml IL-2 after subtracting the levels of IL-2 resolved in the control well (179 pg). Both peptides were tested in the same experiment performed in parallel. IL-2 was determined in the same experiment. A, donor 4 (B and C) donors 3 isolated CD8+ cells. αB7.1 and B7.2 mAb were added to the DC 1 h before addition of responders (hatched) indicates levels of IL-2 determined at 24 h from cultures stimulated with either peptide at 20 μg/ml in the presence of αB7.1.

The increase in overall levels of IL-12 following exogenous IL-12 addition was paralleled by increase in levels of IFN-γ in response to both Ag. Thus the role of exogenous IL-12 is to compensate for the insufficient levels of IL-12 secreted by DC at encounter with tumor Ag reactive CD8+ cells (Fig. 5A vs. B). The levels of IL-12 induced by 5 μg M1 were similar with the levels of IL-12 induced by 20 μg E75. At these peptide concentrations IFN-γ was not detected in response to E75. Therefore, the results indicate that there is a minimum required level of endogenous IL-12 to be present in cultures for IFN-γ to be detected in response to tumor Ag. When IL-12 is below this level, IFN-γ cannot be detected in response to peptide stimulation (Fig. 5A and C). This deficiency was compensated sometimes by high concentrations (>100 μg/ml) of E75, (Figs. 1E and 4A), which induced substantially higher levels of IL-12 (>1,600 pg/ml). (not shown).

Primary stimulation with E75 induced IL-2 in healthy donor CD8+ cells. Enhancement by αB7.1. Regulation of T-cell response by Ag involves at least two major mechanisms: the first by direct induction of IL-12 from APC, through CD40L-CD40 and the second through the B7-CD28 interaction (22,23). The former apparently controls the IFN-γ induction, while the latter controls the IL-2 secretion and responsiveness to IL-2 through high affinity IL-2R induction (24,25). The first pathway can also positively impact on the second through up-regulation, among others, of costimulatory molecules of the B7 family. A B7-CD28 dependent costimulatory pathway can also mediate a functional type 1 cytokine response (26,27) and synergized with IL-12 (28). Results in Figs. 1C and 3C show that α-CTLA-4 enhanced IFN-γ induction in response to E75 + IL-12. This suggested that the responders may be activated but negative signaling after ligation of B7 reduces the response. If this is the case, blocking of B7 is expected to reverse the inhibitory effects. This will be evidenced by enhanced induction of IFN-γ, IL-2 and proliferation. To directly address the role of B7 in E75-induced cytokines, we investigated the role of B7-1 in IFN-γ and IL-2 induction.

To verify that induction of IFN-γ by E75 is enhanced by B7 blocking, the experiment was repeated with donor 4 using isolated CD8+ cells. Since B7.1 and B7.2 were expressed on DC at different levels, we used alternatively αB7.1 and αB7.2 mAb to block the receptor ligation. In the absence of IL-12, αB7.1 did not enhance IFN-γ in response to E75, while αB7.2 co-stimulated IFN-γ induction only weakly (=50 pg/ml). However, in the presence of IL-12, αB7.1 enhanced the E75 + IL-12 induced response by two fold. The potentiating effect of B7.2 was much weaker. The results (Fig. 6A) confirmed that the synergy between E75 and αB7 for high IFN-γ secretion within the first 24 h required IL-12. Since blocking of B7-CTLA4 synergized with Ag + IL-12 in IFN-γ production these results confirmed that the responders were activated CD8+ cells.

To characterize the ability of E75-reactive CD8+ cells to produce IL-2 the experiment was repeated with donor 3 using M1 as positive control. Since in activated T-cells blocking of B7/CTLA-4 was reported to reverse the state of tolerance of T-cells for proliferation through induction of IL-2 (29), we investigated whether in our system B7.1 was required for IL-2 induction in response to E75 and M1. E75 and M1 were used at the same concentrations (5 and 20 μg/ml) as for IL-12 and IFN-γ induction in the experiment shown in Fig. 5. E75 induced IL-2 in a concentration-dependent manner in the absence of αB7.1. The IL-2 levels at 5 and 20 μg/ml E75 were 4 and 2-fold lower, respectively, than the levels induced by M1. Twenty μg E75 induced the same levels of IL-2 as 5 μg M1. αB7.1 did not inhibit IL-2 production, but in contrast it had a slight enhancing effect by 20% (E75) and 40% (M1) compared with peptide alone. Even in the presence of αB7.1 the levels of IL-2 induced by M1 were at least two fold higher than the levels induced by E75 (Fig. 6B). Thus previously *in vivo* activated CD8+ cells in this healthy donor are not tolerized/ anergic with respect to IL-2 production.

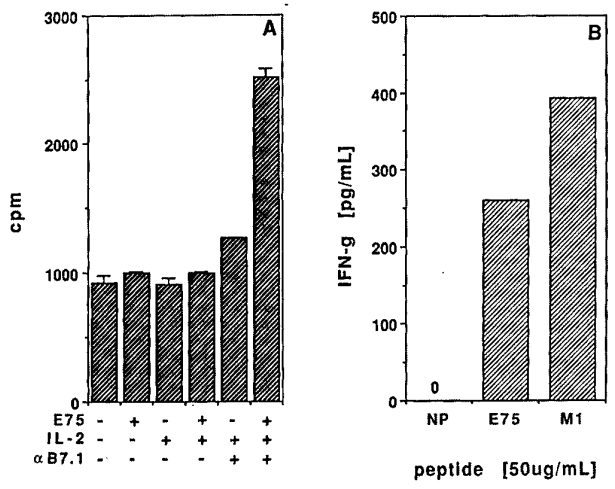

Figure 7. A, Proliferative responses of CD8+ populations. αB7.1 enhanced the rate of proliferation induced by priming with E75 in the presence of IL-2. CD8+ cells from donor 3 were stimulated with DC-E75 for 72 h. 10^5 cells were collected from each well, and pulsed with ^{3}H-TDR for the last 8 h. All measurements were performed in tetraplicate. Results indicate cpm/50,000 cells ± SD. B, IFN-γ response of preactivated donor 3 CD8+ cells. CD8+ cells primed with DC-E75 + IL-12 and maintained in culture for a total of 5 days were rested for 24 h in the absence of IL-2 and restimulated on day 6 with DC-E75 without exogenous IL-12. Supernatants were collected at 20 h. Results indicate pg/ml/10^6 cells.

Since IL-2 acts as a survival factor, exogenous IL-2 at 200 pg/ml was added for the last 8 h before supernatant collection in a parallel experiment. The results in Fig. 6C show the IL-2 levels induced by E75α, M1 ± αB7.1, after correction by subtracting the amount of exogenous IL-2 recovered in the control wells. The levels of IL-2 induced by E75, increased by two fold compared with the levels of IL-2 induced by E75 in IL-2 absence. In the same conditions the levels of IL-2 induced by M1 increased by 5-fold. αB7.1 increased significantly, by 2.5-fold, the levels of IL-2 induced by E75 but less, by 1.5 the levels of IL-2 induced by M1 without αB7.1. Again, levels of IL-2 induced by 20 μg E75 + αB7.1 were similar with the levels induced by 5 μg M1. The IL-2 response in E75-stimulated cells was stable for the next 24 h. At 48 h, the IL-2 levels for 5 and 20 μg E75 were 88 and 120 pg/ml respectively. Thus, when *in vivo* E75-activated cells remain viable they are able to produce more IL-2 in response to blocking of B7.1 confirming that they are not anergic.

Priming with E75 + B7.1 + IL-2 weakly increased CD8+ cells proliferation (Fig. 7A) similarly with E75 + CTLA-4 (Fig. 3D). This suggested that these cells undergo only limited expansion in response to E75. E75 + IL-2 + αB7.1 induced weak proliferation at 72 h, stimulation index (SI) = 2.53. IL-2 + αB7.1 without E75 induced borderline proliferation (SI = 1.28) compared with control E75-stimulated cultures: (SI = 1.0, i.e., 993 cpm ± 50). Thus, in the presence of IL-2 with blocking of B7.1, CD8+ cells responded to the E75 signal better than in the absence of αB7.1. Thus the negative signaling through B7-CTLA4 could provide a mechanism by which the proliferation of these cells is inhibited.

To address the possibility that the cells were not becoming non-responsive as a result of Ag activation, and required IL-12 as a consequence of cytokine-mediated tolerization, E75 + IL-12 and M1 + IL-12 primed cells from donor 3 were restimulated with E75 and M1 respectively in the absence of IL-12. IFN-γ was determined 20 h later. The results in Fig. 7B show that on a per cell basis these cells responded to Ag stimulation, in the absence of IL-12, with even higher levels of IFN-γ. Thus CD8+ cells present in healthy donors, after *in vitro* stimulation became more responsive to restimulation through the TCR, regarding proliferation and IFN-γ production, suggesting that under these stimulation conditions the Ag is not tolerogenic.

Primary stimulation with DC - E75 ± IL-12, αB7/CTLA-4 induce Ag-specific.cytolytic activity infrequently. Ag-specific CTL activity was observed at priming only in two donors and inconsistently over time in two other donors. This suggested that stimulation of this function required different activation thresholds, which could not be reached by DC-E75. Ag-specific CTL activity was detected in two other donors after 3-4 stimulations suggesting that differences in frequency together with the slow rate of division of these cells did not allow CTL effectors to reach the critical numbers for specific lysis to become evident (Anderson *et al*, preliminary data). These results show that activated tumor Ag reactive CD8+ cells are present in healthy individuals and are not tolerized. They are responsive to stimulation through the TCR since they rapidly secrete IL-2, IFN-γ and IP-10 (Lee *et al*, preliminary data) as well as induce IL-12 in DC. Tumor Ag such as E75, are not tolerogenic but weak inducers of IL-2 and IL-12 and even weaker inducers of proliferation, suggesting that they are weak/partial agonists for activation of effector functions.

Discussion

In this study, we investigated the presence of activated, tumor Ag- and tumor-reactive cells in CD8+ cells freshly isolated from PBMC of healthy donors. The IFN-γ, IL-2, and IL-12 induced by E75 and E75-reactive CD8+ cells mirrored the response to M1 conventially used to define the presence of activated CD8+ influenza specific cells in healthy individuals. We found that E75-reactive CD8+ cells are present in some donors in a state wherein they can secrete IFN-γ within 6-12 h of antigen exposure. Our results show that T-cells reactive with HER-2, FBP and AES peptides exist in healthy donors and they are rapidly activated by tumor Ag in a similar fashion with the memory cells reactive with viral Ag. The fact that in the same donor Ag induced IFN-γ was detectable at different time points in the absence or presence of IL-12 may reflect changes in the numbers of E75-specific cells or in their state of activation. Since the IFN-γ response was obtained primarily from CD45RO+ cells, and was amplified by α-CTLA4/αB7 only in the presence of IL-12, while the IL-2 response was amplified by αB7.1, our results suggest that E75-reactive activated CD8+ cells are frequently present in healthy individuals.

In most donors, high IFN-γ induction by Ag was detected within 20 h when costimulated by IL-12. The exogenous IL-12 requirement for detection of IFN-γ was dependent on the Ag

sequence and concentration. The sequence dependency for peptides derived from the same protein was supported by the fact that in the same donor the IFN-γ responses to other HER-2 peptides of higher (F57) or weaker (GP2/F53) binding affinity to HLA-A2 were significantly lower than to E75. This also raised the possibility that $CD8^+$ cells of different affinities for each tumor Ag and at different frequencies may be present in the same individual. This was suggested by the facts that: i) IFN-γ responses to FBP peptide E41 and to the AES peptide G76 both of lower HLA-A2 affinity than E75 showed similar or higher IFN-γ levels with responses to E75 and ii) at high (100-150 μg/ml) E75 concentration exogenous IL-12 was not required for IFN-γ induction. IL-12 was required when E75 was used at 5-20 μg/ml. Similarly, influenza-matrix, M1-reactive-$CD8^+$ cells from the same donor required exogenous IL-12 when stimulated with 5 μg but not with 20 μg of M1. For both Ag (E75 and M1) the requirement for exogenous IL-12 for IFN-γ induction inversely correlated with the amount of endogenous IL-12 induced.

The presence of activated $CD8^+$ cells specific for E75 in the PBMC of healthy donors is also supported by the fact that these cells induced rapidly IL-12 from APC. IL-12 production increased in direct proportion to E75 concentration. Since induction of IL-12 require CD40 triggering, only when T-cells are involved (24), these results support the possibility that the responders to E75 and the other tumor Ag are activated $CD8^+$ cells. We found that the amount of IL-12 induced in APC by peptide-reactive $CD8^+$ cells should be above a certain level for IFN-γ to be detected in response to E75/M1. In the donors studied E75, at concentrations as high as 20 μM cannot induce sufficiently high levels of IL-12 required for mediation of IFN-γ costimulatory activity. The IL-12 dependent control of IFN-γ response to Ag may provide a mechanism for maintaining these cells in a non-responsive state. This may reflect the requirement for higher levels of signaling by E75 and the other tumor Ag (AES, FBP) for TCR-mediated activation of the existent reactive $CD8^+$ cells compared with conventional Ag (30). By comparing the levels of IL-12 and IL-2 induced by E75 and M1 at two Ag concentrations it appeared that M1 is at least 4-fold more potent than E75 in cytokine induction. Thus the lack of IFN-γ at low E75 concentrations did not reflect poor Ag presenting/T-cell-activating ability by DC used as APC.

To gain insight into the requirements for activation of these cells we investigated the mechanisms of activation that may be affected by E75 recognition. IL-12 production is amplified either by increased CD40L expression on T-cells or by endogenous IFN-γ production. Both pathways are dependent on Ag concentration (24). Although E75 stimulation increased CD40L levels on T-cells it did not increase the numbers of $CD40L^+$ cells. Using two-color FACS analysis (CD40L-PE vs. CD8-FITC) we found that at 20 h E75 stimulation increased the vertical (Y) mean for $CD40L^+$ $CD8^+$ cells in the upper right quadrant by three fold (from 13.3 to 34.8). Addition of IL-12 doubled the Y mean level to 70.9, but not the % positive cells. The $CD8^+$ $CD40L^+$ cells were in the range 0.2-0.3%. We also determined the expression of IL-2Rα (CD25), an indicator of responsiveness through TCR, on E75-stimulated cells. Stimulation with E75 + IL-2 increased only weakly the Y mean for $CD8^+$ IL-$2R\alpha^+$ cells, from 23.2 in control (no peptide + IL-2) to 27.2 in E75 + IL-2. Thus E75 appeared to have distinct potencies for induction of CD40L and IL-2Rα.

We demonstrated that IL-12 induction by E75 was not dependent on the presence of endogenous IFN-γ (Fig. 5). However, low (50 pg) of exogenous IFN-γ amplified the IL-12 response to E75. Therefore, low levels of IFN-γ induced by pathogens, or crossreactive Ag in the vicinity of these cells, *in vivo* may activate IFN-γ production by tumor Ag in these cells through a positive feedback loop: Ag1 + low IFN-γ→IL-12→IL-12 + Ag2→more IFN-γ. The exogenous IL-12 requirement for IFN-γ activation may be due to the fact that E75 is a weak inducer of CD40L. Low levels of CD40L in E75-reactive memory cells cannot induce the minimal levels of IL-12 to co-stimulate for IFN-γ production in response to E75. Another possibility which was not yet investigated but deserve attention in further studies is that the signal transduced by E75 in T-cells is also a weak inducer of the IL-12Rß chain, which is required for the high affinity IL-12Rß expression. The high affinity IL-12R increase the sensitivity of responders to lower concentrations of IL-12, and it is stabilized by IFN-γ (31).

Given our data demonstrating a role for CD40L and IL-12 in regulating IFN-γ induction from E75-reactive $CD8^+$ cells and the reports that IFN-γ induction and IL-12 responsiveness in T-cells can be also enhanced via CD28 we studied the involvement of B7-costimulation in IFN-γ and IL-2 production. The regulation of IFN-γ induction by IL-12 was confirmed by IFN-γ enhancement by mAb to B7/CTLA-4 only in the presence of IL-12.

An enhancing role for blocking of B7 was observed in IL-2 induction by E75. E75 alone induced IL-2 within 24 h, although at low levels. α-B7.1 increased E75-induced IL-2. The fold increase was higher for E75 than for M1 in the presence of IL-2. This suggested that E75-reactive $CD8^+$ cells do not differ from positive control, *in vivo* activated matrix-reactive $CD8^+$ cells, in their ability to induce/secrete: IL-12, IL-2 and IFN-γ. They differed in the overall amounts of cytokines secreted. Since 4-fold more E75 was required to induce the same levels of IL-12, IFN-γ, and IL-2, induced by M1, this raises the possibility that the activated $CD8^+$ cells are in a hyporesponsive state. Since these results were obtained only with few donors, additional studies using separated populations are required to address this point. The 2-4-fold difference in cytokine levels may not exclude the possibility that the frequency of E75-specific CTL is lower than the frequency of M1-specific CTL, or that within the E75-responders there are subpopulations endowed with high IFN-γ secretion activity.

Priming with E75 in the presence of IL-2, αCTLA-4 or αB7.1 although enhanced IL-2 production increased only marginally the responders proliferation. The reasons for this selective responsiveness are unknown. E75 + IL-12 primed cells secreted high levels of IFN-γ at restimulation with E75. Thus they maintained responsiveness through the TCR. However, their poor proliferative ability was not reversed by preculture in IL-2 as was expected if they were partially tolerized/anergic (Lee *et al*, preliminary data).

One possibility to be considered is that this functional dichotomy reflects a weak agonistic activity of E75 in that

the signal transduced through TCR can activate the IFN-γ, IL-12, IL-2 (this paper) and IP-10 induction (Lee *et al,* preliminary data) but sustains a slow division of these cells. The outcome of this slow division is that the frequency of specific cytolytic effectors increase slowly with each Ag stimulation. Based on $CD8^+$ $CD40L^+/IL\text{-}2R\alpha^+$ data the frequency of these cells is less than 10-3 but not less than 1/10,000-1/20,000. If a minimum frequency of 10^{-1}-10^{-2} is required for detection of specific CTL activity (at E:T = 10-20/1) (15), and these cells increase in number by 4-5-fold after 2 stimulations, to reach the minimum threshold of 10-fold increase will require more than 3 stimulations. This is in agreement with reports on restimulation requirements for induction of tumor cytolysis (32). This is also supported by the slow increase in lytic units specific for melanoma Ag in healthy donors compared with cancer patients (4). In support of our conclusions, it has been recently shown that: i) acquisition of cytotoxic function by activated CTL require at least one cell division (33) and ii) T-cells that survive as memory cells proliferate weakly during the expansion phase of an immune response (34).

Our work raised the intriguing possibility that activated HER-2 Ag-specific $CD8^+$ cells exist *in vivo* in healthy individuals. This would make sense since it is unclear how a peripheral repertoire of naive T-cells specific for tumor Ag could be maintained in healthy donors in the absence of Ag stimulation, and how it can survive only on cytokines secreted in *trans* by other cells. Their hyporesponsive state may be due to the increased threshold for self-reactivity as recently described in IEL from self-Ag^+ mice (35). This hyporesponsitivity may reflect the weak immunogenicity of the tumor Ag in the periphery for positively selected self-reactive cells (reviewed in ref. 36). Epithelial tissues expressing HER-2 or FBP may provide the epitope precursors and/or crossreactive immunogens due to physiological turnover, while IL-12 induced by pathogens at the same time may costimulate in *trans* IFN-γ secretion. Similarly, IL-2 from pathogen stimulated $CD4^+$ cells can drive their expansion or act as survival factor. Activated $CD8^+$ cells may not need professional APC for activation, since the B7-CTLA4 interaction may be in fact inhibitory. These populations may be maintained in steady-state by death of activated cells (AICD) expressing higher-affinity receptors for Ag. The affinity for the tumor Ag of the surviving population may decline over time. The surviving cells may be reactivated as CTL-TAL only when the tumor expresses very high concentrations of Ag (e.g., HER-2, gp100, FBP). At that time, tumor-derived IL-10, TGF, angiogenic chemokines, will inhibit the functional IL-12, IFN-γ and IP-10 response.

Identification of activated tumor-reactive memory $CD8^+$ cells in healthy individuals with high frequency raise several novel and possibly important implications for tumor immunosurveillance, and vaccine design: i) since such cells are present in healthy donors their rapid mobilization to mediate cytokine associated effector functions may be useful for maintaining a mechanism of control of tumor emergence in individuals at high risk or in patients with no evidence of disease. This may be achieved by periodic stimulation with low concentrations of tumor Ag plus IL-12. This should be particularly relevant for ovarian cancer where the recurrence rate is high. ii) The sensitivity of IL-12 induction and of the IL-12R to negative regulation by IL-10, may require, in cancer patients, approaches to neutralize regulatory cytokines at vaccination with tumor Ag if the activation of IFN-γ^+ effectors is aimed. ii) Naive $CD8^+$ cells primed by tumor Ag plus B7 costimulation, over time convert to memory cells. After death, due to repeated encounters with Ag, *in vivo*, the surviving cells of lower affinity for Ag will require even higher Ag concentrations for activation than at priming. Blocking of B7-CTLA4 will be unable to reverse negative regulation of CTL expansion if the tumor Ag cannot reach the threshold for TCR signaling for cell cycling. Thus, the weak signaling demonstrated by the wild-type tumor Ag would require development of different TCR agonists than the ones currently used, for activation of proliferation and rescue of the high affinity memory cells, to be used for vaccination. Therefore, the results reported above may be useful to develop approaches to activate cellular immunity to tumors.

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References

1. Houghton AN: Cancer antigens: immune recognition of self and altered self. J Exp Med 180: 1-4, 1994.
2. Pittet M, Valmori D, Dunbar PR, Speiser DE, Lienard D, Lejeune F, Fleischhauer K, Cerundolo V, Cerottini J-C and Romero P: High frequencies of naive melan-A/MART-1-specific $CD8^+$ T cells in a large proportion of human histocompatibility leukocyte antigen (HLA)-A2-individuals. J Exp Med 190: 705-715, 1999.
3. Anichini A, Molla A, Mortarini R, Tragni G, Bersani I, DiNicola M, Gianni AM, Pilotti S, Dunbar R, Cerundolo V and Parmiani G: An expanded peripheral T cell population to a cytotoxic T lymphocyte (CTL)-defined, melanocyte-specific antigen in metastatic melanoma patients impacts on generation of peptide-specific CTLs but does not overcome tumor escape from immune surveillance in metastatic lesions. J Exp Med 190: 651-667, 1999.
4. Marincola FM, Rivoltini L, Salgaller ML, Player M and Rosenberg SA: Differential anti-MART-1/MelanA CTL activity in peripheral blood of HLA-A2 melanoma patients in comparison to healthy donors: evidence of *in vivo* priming by tumor cells. J Immunother 19: 266-277, 1996.
5. Goldrath AW and Bevan MJ: Selecting and maintaining a diverse T-cell repertoire. Nature 402: 255-262, 1999.
6. Fisk B, Blevins TL, Wharton JT and Ioannides CG: Identification of an immunodominant peptide of HER-2/neu protooncogene recognized by ovarian tumor-specific cytotoxic T lymphocyte lines. J Exp Med 181: 2109-2117, 1995.
7. Brossart P, Stuhler G, Flad T, Stevanovic S, Rammensee HG, Kanz L and Brugger W: Her-2/neu-derived peptides are tumor-associated antigens expressed by human renal cell and colon carcinoma lines and are recognized by *in vitro* induced specific cytotoxic T lymphocytes. Cancer Res 58: 732-736, 1998.
8. Rongcun Y, Salazar-Onfray F, Charo J, Malmberg KJ, Evrin K, Maes H, Kono K, Hising C, Petersson M, Larsson O, Lan L, Appella E, Sette A, Celis E and Kiessling R: Identification of new HER2/neu-derived peptide epitopes that can elicit specific CTL against autologous and allogeneic carcinomas and melanomas. J Immunol 163: 1037-1044, 1999.

9. Peoples GE, Goedegebuure PS, Smith R, Linehan DC, Yoshino I and Eberlein TJ: Breast and ovarian cancer-specific cytotoxic T lymphocytes recognize the same HER2/neu-derived peptide. Proc Natl Acad Sci USA 92: 432-436, 1995.
10. Peoples GE, Anderson BW, Fisk B, Kudelka AP, Wharton JT and Ioannides CG: Ovarian cancer-associated lymphocyte recognition of folate binding protein peptides. Ann Surg Oncol 5: 743-750, 1998.
11. Babcock B, Anderson BW, Papayannopoulos I, Castilleja A, Murray JL, Stifani S, Kudelka AP, Wharton JT and Ioannides CG: Ovarian and breast cytotoxic T lymphocytes can recognize peptides from the amino-enhancer of split protein of the Notch complex. Mol Immunol 35: 1121-1133, 1998.
12. Miller C, Ragheb JA and Schwartz RH: Anergy and cytokine-mediated suppression as distinct superantigen-induced tolerance mechanisms *in vivo*. J Exp Med 190: 53-64, 1999.
13. Bird JJ, Brown DR, Mullen AC, Moskowitz NH, Mahowald MA, Sider JR, Gajewski TF, Wang C and Reiner SL: Helper T cell differentiation is controlled by the cell cycle. Immunity 9: 229-237, 1998.
14. Deeths MJ, Kedl RM and Mescher MF: $CD8^+$ T cells become non-responsive (anergic) following activation in the presence of costimulation. J Immunol 163: 102-110, 1999.
15. Lalvani AR, Brookes S, Hambleton WJ, Britton AVS, Hill and McMichael AJ: Rapid effector function in $CD8^+$ memory T cells. J Exp Med 186: 859-865, 1997.
16. Perez VL, Parijs LV, Biuckians A, Zheng XX, Strom TB and Abbas AK: Induction of peripheral T cell tolerance *in vivo* requires CTLA-4 engagement. Immunity 6: 411-417, 1997.
17. Sallusto F and Lanzavecchia A: Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor α. J Med Exp 179: 1109-1118, 1994.
18. Pickl WF, Majdic O, Kohl P, Stockl J, Riedl E, Scheinecker C, Bello-Fernandez C and Knapp W: Molecular and functional characteristics of dendritic cells generated from highly purified $CD14^+$ peripheral blood monocytes. J Immunol 157: 3850-3859, 1996.
19. Fisk B, Hudson JM, Kavanagh J, Wharton JT, Murray JL, Ioannides CG and Kudelka AP: Existent proliferative responses of peripheral blood mononuclear cells from healthy donors and ovarian cancer patients to HER-2 peptides. Anticancer Res 17: 45-53, 1997.
20. Barbulescu K, Becker C, Schlaak JF, Schmitt E, zum Buschenfelde KM and Neurath MF: Cutting Edge: IL-12 and IL-18 differentially regulate the transcriptional activity of the human IFN-γ promoter in primary $CD4^+$ T lymphocytes. J Immunol 160: 3642-3647, 1998.
21. Gollob JA, Schnipper CP, Murphy EA, Ritz J and Frank DA: The functional synergy between IL-12 and IL-2 involves p38 mitogen-activated protein kinase and is associated with the augmentation of STAT serine phosphorylation. J Immunol 162: 4472-7781, 1999.
22. Gately MK, Renzetti LM, Magram J, Stern A, Adorini L, Gubler U and Presky DH: The interleukin-12/interleukin-12-receptor system: role in normal and pathologic immune responses. Annu Rev Immunol 16: 495-521, 1998.
23. Walunas TL, Bakker CY and Bluestone JA: CTLA-4 ligation blocks CD28-dependent T cell activation. J Exp Med 183: 2541-2550, 1996.
24. DeKruyff RH, Gieni RS and Umetsu DT: Antigen-driven but not lipopolysaccharide-driven IL-12 production in macrophages requires triggering of CD40. J Immunol 158: 359-366, 1997.
25. Willerford DM, Chen J, Ferry JA, Davidson L, Ma A and Alt FW: Interleukin-2 receptor α chain regulates the size and content of the peripheral lymphoid compartment. Immunity 3: 521-530, 1996.
26. McDyer JF, Goletz TJ, Thomas E, June CH and Seder RA: CD40 ligand/CD40 stimulation regulates the production of IFN-γ from human peripheral blood monuclear cells in an IL-12- and/or CD28-dependent manner. J Immunol 160: 1701-1707, 1998.
27. Yang Y and Wilson JM: CD40 ligand-dependent T cell activation: requirement of B7-CD28 signaling through CD40. Science 273: 1862-1864, 1996.
28. Walker W, Aste-Amezaga M, Kastelein RA, Trinchieri G and Hunter CA: IL-18 and CD28 use distinct molecular mechanisms to enhance NK cell production of IL-12-induced IFN-γ. J Immunol 162: 5894-5901, 1999.
29. Krummel MF and Allison JP: CTLA-4 engagement inhibits IL-2 accumulation and cell cycle progression upon activation of resting cells. J Exp Med 183: 2533-2540, 1996.
30. Hemmer B, Stefanova I, Vergelli M, Germain R and Martin R: Relationships among TCR ligand potency, thresholds for effector function elicitation, and the quality of early signaling events in human T cells. J Immunol 160: 5807-5814, 1998.
31. Szabo SJ, Dighe AS, Gubler U and Murphy KM: Regulation of the interleukin (IL)-12Rα2 subunit expression in developing T helper 1 (Th1) and Th2 cells. J Exp Med 185: 815-824, 1997.
32. Perez-Diez A, Butterfield LH, Li L, Chakraborty NG, Economou JS and Mukherji B: Generation of $CD8^+$ and $CD4^+$ T-cell response to dendritic cells genetically engineered to express the MART-1/Melan-A gene. Cancer Res 58: 5305-5309, 1998.
33. Oehen S and Brduscha-Riem K: Differentiation of naive CTL to effector and memory CTL: correlation of effector function with phenotype and cell division. J Immunol 161: 5338-5346, 1998.
34. Jacob J and Baltimore D: Modelling T-cell memory by genetic marking of memory T cells *in vivo*. Nature 399: 593-597, 1999.
35. Guehler SR, Rosalynde JF, Bluestone JA and Barrett TA: Increased threshold for TCR-mediated signaling controls self reactivity of intraepithelial lymphocytes. J Immunol 160: 5341-5346, 1998.
36. Germain RN and Stefanova I: The dynamics of T cell receptor signaling: complex orchestration and the key roles of tempo and cooperation. Annu Rev Immunol 17: 467-522, 1999.